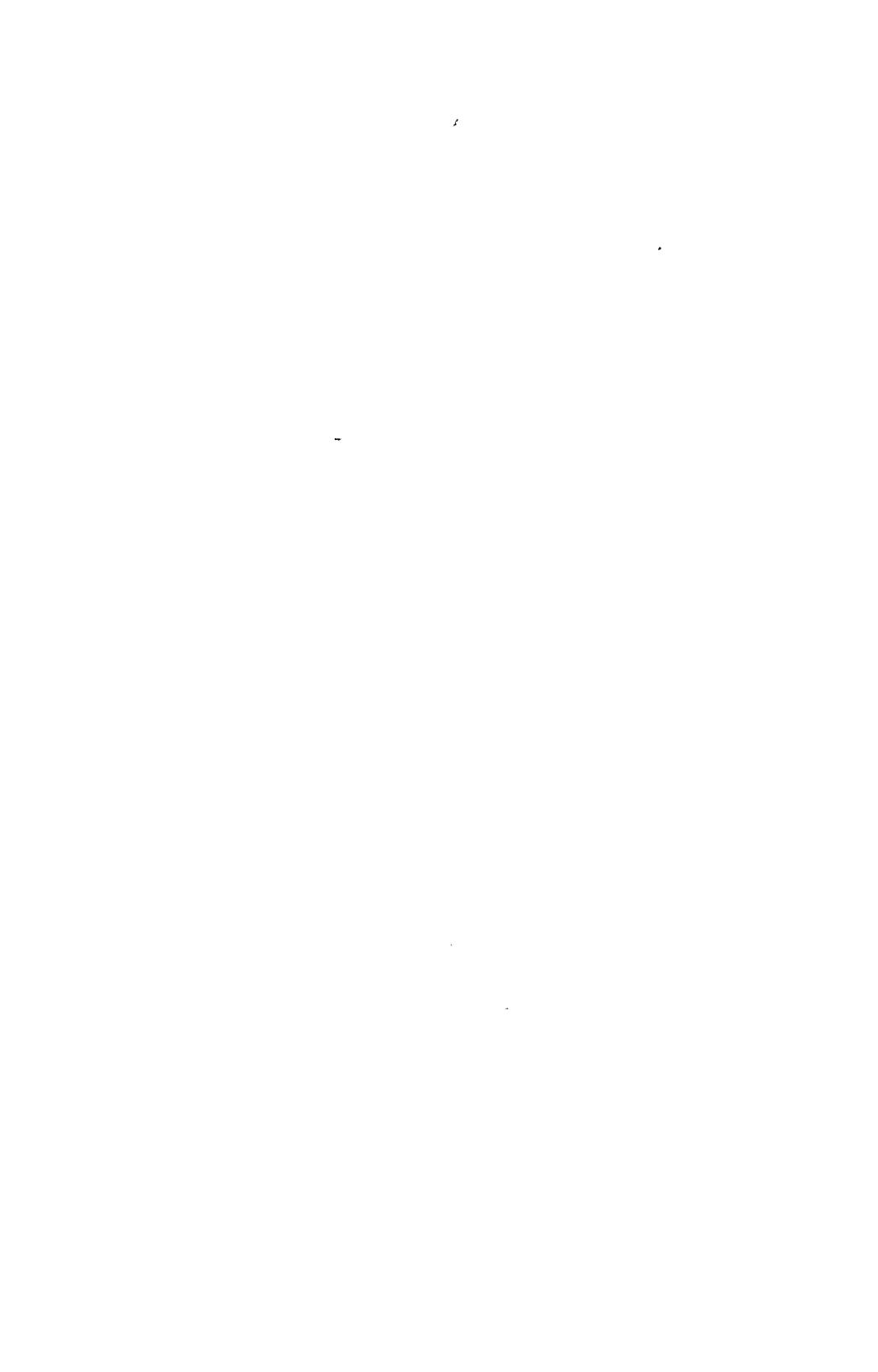




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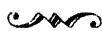
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CEREAL CHEMISTRY

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No. 1

PREPARATION OF AMYLASE-ACTIVE CONCENTRATES FROM MOLD BRAN¹

ROBERT L. GATES² and ERIC KNEEN³

ABSTRACT

In the investigation of the precipitation potentialities of methanol, ethanol, isopropanol, and acetone, it was ascertained that under the proper conditions any of these compounds may be used for amylase precipitation. Methanol requires low temperature (0°C.) operations to obtain satisfactory recovery, while this is not mandatory for the other three compounds. Arranged in order of precipitation efficiency, based on the concentration required to give 90% recovery of amylase activity, they fall into the order: acetone, isopropanol, ethanol, methanol. Methanol and ethanol cause loss of activity when allowed to remain in contact with the enzyme precipitate either in the precipitation operation or in drying. Isopropanol and acetone do not show this property. Separation may be aided by the presence of certain cations. With all precipitants optimum hydrogen ion concentration for precipitation is pH 5.5-6.5. The presence of an excess of multi-valent cations in the extract is detrimental to the activity of the precipitate. Isopropanol is the most tolerant to the presence of these ions.

The ability of certain organic substances to precipitate the enzymes of mold bran from a water extract is well established, but the influence of certain environmental factors upon the enzyme activity and upon the physical characteristics of such precipitates is not so well known. Additional information on these factors not only should help in the commercial processing of mold bran but also should provide valuable information relative to the fundamental properties of the mold amylases.

Many approaches have been made to the problem of purification of the amylases. The majority of the work done in this field has been to obtain a relatively pure concentrate for further studies of the properties of the enzyme.

¹ This paper represents a portion of a thesis presented to the Graduate School of Kansas State College in partial fulfillment of the requirements for the degree of Master of Science. The work was supported by a grant from the Farm Crops Processing Corporation. Contribution No. 143, Department of Milling Industry. Manuscript received October 8, 1947.

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One of the first references to a purification method for amylases is the report of Payen and Persoz (11) that extracts of malt were precipitated with ethanol to concentrate the active principle. A great deal of work has since been done on the use of ethanol as a precipitating agent. Newton and Naylor (10) used 65% ethanol to precipitate the amylase from soybean extracts. The work of Sherman and Schlesinger (14) shows the feasibility of ethanol as a precipitating agent for the concentration of pancreatic amylases. The differential solubility in ethanol of the alpha and beta amylases of malt has been used by numerous workers to obtain relatively pure alpha and beta amylase. The background of this application has been discussed by Kneen, Sandstedt, and Hollenbeck (8). Ethanol has likewise been employed for the purification of mold amylases (17, 3, 9).

The importance of acetone as a precipitating agent can be inferred from its incorporation in the purification methods of Sherman and Schlesinger (15) and Tilden, Adams, and Hudson (19).

The work of Hayasi (4) suggests the use of methanol as a precipitating agent for mold amylases.

As an aid to precipitation of mold amylases the addition of barium chloride to the enzyme infusion prior to precipitation with ethanol is suggested by Takatomi and Takeda (18).

A detrimental effect of ethanol on the activity of the precipitate was reported by Sherman and Schlesinger (16), and Blish, Sandstedt, and Mecham (2).

The present study was prompted by the paucity of information available on the details for the separation of amylase from mold brans and the precipitation of these enzymes in an active, water-soluble, and concentrated form. Such information is a prerequisite to commercial application of the materials as well as to a better understanding of the fundamental nature of the enzymes.

Materials and Methods

In the investigation of precipitation procedures, four organic compounds have been compared under various conditions as precipitating agents, i.e., methanol, ethanol, isopropanol, and acetone.

A quantity of commercial mold bran was obtained from the Mold Bran Company of Eagle Grove, Iowa, and this was used throughout the experiments as the crude product from which the enzyme was extracted for precipitation. This bran was produced by the growth of a strain of *Aspergillus oryzae* on wheat bran. For comparison of enzyme systems, other commercial mold brans were obtained from Wallerstein Company, Jeffreys Laboratories, and Jacques Wolfe Company. Fungal concentrates from Rohm and Haas (RHozyme S),

Wallerstein Company, and Schwarz Laboratories (Polidase S) also were used in some of the comparisons.

The principal amylase in fungal enzyme systems being of the alpha type as pointed out by Kneen and Sandstedt (7), a modified Wohlgemuth (20) method was used to follow precipitation recoveries. This method is based on the time required to obtain the red-brown color with iodine described by Sandstedt, Kneen, and Blish (13). A known amount of extract was allowed to digest a 20-ml. aliquot of 1% boiled soluble starch buffered with sodium citrate-hydrochloric acid buffer at a pH value of 5.0. The time (dT) in minutes required by an appropriate aliquot to convert the substrate at 30°C. to a point where the "red-brown" color was given with iodine was determined.

The aliquots of the extracts or solutions used for activity determinations varied from 2 to 10 ml. depending on activity. Since the total reaction volume was constant, 30 ml., it was necessary to adjust the volume with 0.2% calcium chloride solution (Hollenbeck and Blish, 5), when less than 10 ml. enzyme aliquot were employed. The accuracy of this procedure is about 5%, so only differences in results of this order or greater are considered significant.

The method outlined by Kneen and Beckord (6) was used for comparison of the saccharification action of the various enzyme systems. This method measures the comparative amount of fermentable sugars produced. A starch substrate was digested by the enzyme, the sugars produced fermented by yeast, and this fermentation followed manometrically in the "pressure meter" of Sandstedt and Blish (12).

In order that a greater number of precipitation factors might be studied it was deemed desirable to use a simple and rapid method of following precipitation recovery. Ten ml. of a one to ten extract (one part mold bran to ten parts extractant) of the mold bran were placed in a 100-ml. centrifuge tube and adjusted to the desired set of conditions for precipitation. To this was added a calculated amount of precipitating agent. The precipitate formed was centrifuged out and the supernatant liquid discarded. To the residue were added 40 ml. of distilled water. The dextrinizing activity of an aliquot of this solution was compared with a similar quantity of the original extract, and from this was calculated the per cent recovery. In all subsequent references to recovery of amylase it should be kept in mind that the recovery of amylase was measured in terms of the dextrinizing activity of the redissolved precipitate.

Experimental

After standardizing a method of extraction, the problem of preparing an enzyme concentrate became a process of determining, step

by step, the effect of precipitant concentration, temperature, hydrogen-ion concentration, and the kind and concentration of salt present during precipitation on the activity and physical characteristics of the precipitates.

Extraction of Amylase. The studies on the technique of extraction showed that the total amount of enzyme extracted is constant regardless of the ratio of extraction, but the amount of liquid extract recovered varies inversely with the bran-water ratio in extraction. It was concluded that an extraction ratio of one to ten was most satisfactory for the research. Dilute calcium chloride solutions have been suggested as appropriate extractants for the alpha type of amylase (5). With the particular mold bran used the activity of the extract seemed to be independent of the presence of calcium chloride in the extraction medium. Unless otherwise indicated, all mold bran extractions were made with distilled water.

Three methods of extraction were investigated. Method one was the standard method of Sandstedt, Kneen, and Blish (13). The extraction mixture was allowed to stand for an hour at 30°C. with agitation at 15-minute intervals. Method two made use of a mechanical laboratory stirrer, the extraction mixture being in a beaker in a 30°C. bath. Method three employed a Ward's Liqui-Mixer. A greater concentration of enzyme was obtained from a 3-minute extraction with the Liqui-Mixer than with a 1-hour extraction by the standard method. Fifteen-minute extraction with method two was equal to a 1-hour standard extraction.

A temperature of 30°C. was the most convenient temperature to use, and since satisfactory enzyme infusions were obtained at this temperature, it was used as the standard extraction temperature throughout the work.

The procedure used for removing solid material from the extract was to strain the mixture through coarse cloth and then to centrifuge for the removal of the remaining small particles.

Precipitation of the Amylase—Influence of Precipitant Concentration. The effectiveness of four water-miscible organic compounds for the precipitation of amylase from a mold bran extract is shown in Fig. 1. Precipitation temperatures were 20°C. for methanol (the highest temperature permitting appreciable enzyme recovery) and room temperature for the other precipitants.

At no concentration was it possible to obtain complete amylase recovery with methanol at 20°C. Recovery increased with increase in precipitant added up to 75 to 80% methanol concentration but was not improved at higher concentrations. A similar curve was obtained for ethanol but recovery was more nearly complete. It is obvious from

the curves of Fig. 1 that isopropanol was superior to the other two alcohols, since it gave higher recoveries of enzyme at lower concentrations. A further advantage for isopropanol, particularly in differential fractionation studies, is indicated by the very abrupt rise in the enzyme recovery between 50 and 55% concentration of precipitant.

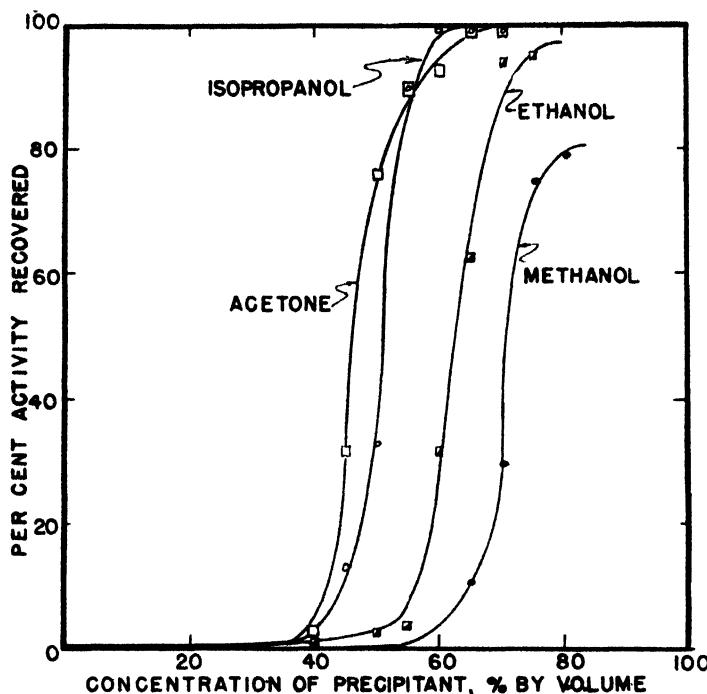


Fig. 1. Effect of precipitant concentration on recovery of amylase activity from mold bran extract.

The data shown for acetone in Fig. 1 demonstrate that it was similar to isopropanol in precipitation properties. However, acetone tended to produce a gummy, discolored precipitate of lower water solubility than that resulting from the alcohol precipitations.

Influence of Temperature. With the four organic compounds used, the recovery of amylase by precipitation was related to both precipitant concentration and temperature; the lower the temperature the lower was the precipitant concentration required for maximum recovery. This is shown in Table I for methanol and in Fig. 2 for ethanol, isopropanol, and acetone. By reducing the precipitation temperature to 0°C. it was possible to obtain enzyme recoveries of as high as 90% with methanol.

TABLE I

EFFECT OF TEMPERATURE ON THE RECOVERY OF PRECIPITATED AMYLASE WITH METHANOL AS THE PRECIPITATING AGENT

Precipitation temperature (°C.)	Per cent amylase recovered	
	75% methanol	80% methanol
0	90	92
10	82	88
20	75	79

The combined temperature-concentration effect is well illustrated in Fig. 2. For example, at 70% concentration, temperature had little effect on recovery from ethanol precipitation. However, when the precipitant concentration was reduced to 65%, temperatures below

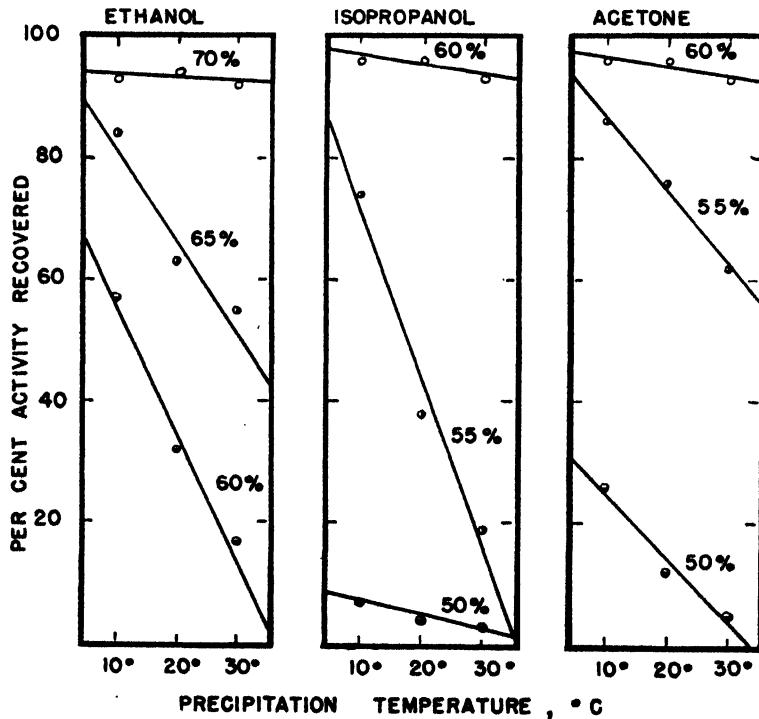


Fig. 2. Effect of temperature and precipitant concentration on recovery of amylase activity from mold bran extract.

10°C. were required to give recovery comparable to that obtained at 70%. The same trend was observed for isopropanol and acetone precipitations. In each instance a minimum precipitant concentration is indicated at which temperature variation up to 30°C. had little

effect on enzyme recovery. Below this minimum the temperature effect was very striking.

Influence of Contact with Precipitant on Precipitate Activity. It was found that enzyme recovery was influenced by the type of precipitant not only in the initial precipitation but also during the period of contact before separation and during the drying of the precipitate. The contact effect during precipitation was determined for methanol and ethanol with distilled water extracts only, and for isopropanol and acetone with both distilled water and dilute calcium chloride extracts of mold bran.

Following methanol precipitations at 20°C. a progressive decrease in enzyme recovery occurred during the time intervening before centrifugation. After 10 minutes standing 69% of the enzyme activity remained; this decreased to 63, 54, and 45% recovery respectively after an additional 1, 2, and 3 hours standing. A similar effect was noted with ethanol precipitation at 30°C. but with consistently higher recoveries of enzyme. After standing 1, 3, and 7 hours before centrifugation, recoveries were respectively 94, 88, and 83%. After 25 hours standing in contact with 70% ethanol at 30°C. only 55% of the enzyme could be recovered.

TABLE II

EFFECT OF THE PRECIPITATING AGENT AND TIME OF CONTACT WITH
PRECIPITANT AT 25°C. ON THE ACTIVITY OF THE AMYLASE
PRECIPITATED FROM WATER EXTRACT AND FROM 0.2%
CaCl₂ WATER EXTRACTS OF MOLD BRAN

Time allowed to stand (min.)	Per cent amylase recovered	
	Isopropanol	Acetone
DISTILLED WATER EXTRACT		
0	100	100
30	100	100
60	93	93
0.2% CALCIUM CHLORIDE EXTRACT		
0	86	96
30	89	75
60	89	65

The data for isopropanol and acetone are given in Table II and demonstrate the high degree of stability of precipitated mold amylase from distilled water extracts. In contrast, the presence of calcium chloride in the extract, even at 0.2% concentration, markedly contributes to low recovery and, with acetone, to an instability of the precipitate.

The loss of enzyme activity during the period necessary for drying the precipitate was next considered. (The precipitate was allowed to

'dry in the bottom of the centrifuge tube.) One series was dried for 2 hours in front of a fan before the activity was determined. Another was dried for 20 hours with no forced air circulation, and the third series was centrifuged and the activity of the precipitate determined immediately. The data shown in Table III indicate more danger of

TABLE III

EFFECT OF DRYING ON THE AMYLASE ACTIVITY FOLLOWING PRECIPITATION WITH 75% METHANOL, 70% ETHANOL, AND 60% ISOPROPANOL AND ACETONE (30°C.)

Time allowed to dry (hrs.)	Per cent amylase recovered			
	75% methanol	70% ethanol	60% isopropanol ¹	60% acetone
0	70	100	88	100
2	69	100	85	100
20	48	83	91	94

¹ Low recovery presumably due to factors other than drying.

loss in enzyme activity from drying methanol and ethanol precipitates than with the other two precipitants.

Influence of Hydrogen-ion Concentration. The difficulty of obtaining complete recovery with methanol and the apparent detrimental effect on the activity of the precipitate discouraged any additional work with this compound, and only ethanol, isopropanol, and acetone were investigated further.

The influence of the hydrogen-ion concentration of the mold bran extract on the recovery of enzyme by ethanol, isopropanol, and acetone precipitation was determined. The pH values were adjusted with hydrochloric acid or sodium hydroxide before addition of the precipitating agent. The concentration of precipitants used was 70% for ethanol, and 60% for both isopropanol and acetone. In all instances the precipitation temperature was 25°C. The data were recorded in terms of per cent enzyme activity recovered at the various pH values and are shown graphically in Fig. 3.

As may be seen from Fig. 3 the optimum hydrogen-ion concentration for ethanol precipitation was between pH 6.0 and 6.5. With isopropanol and acetone the optimum was somewhat lower, being between pH 5.5 and 6.0; either above or below the optimum pH value there was a decrease in enzyme recovery, being very pronounced on the acid side.⁴

⁴ It is appreciated that at least part of the low recoveries on the acid and alkaline sides of the optimum pH value may be attributed to enzyme inactivation. The data of Fig. 3 show the overall effects.

With ethanol precipitation it was observed that with an increase in pH value up to and through the optimum the precipitates became more dense and flocculent. Precipitates formed at pH values in the region of 2.5 to 3.5 remained in suspension and could be separated from the media only by centrifuging. With isopropanol and acetone no effect of pH on rate of precipitate settling was observed—there was very little variation over the pH ranges used, and all precipitates settled slowly.

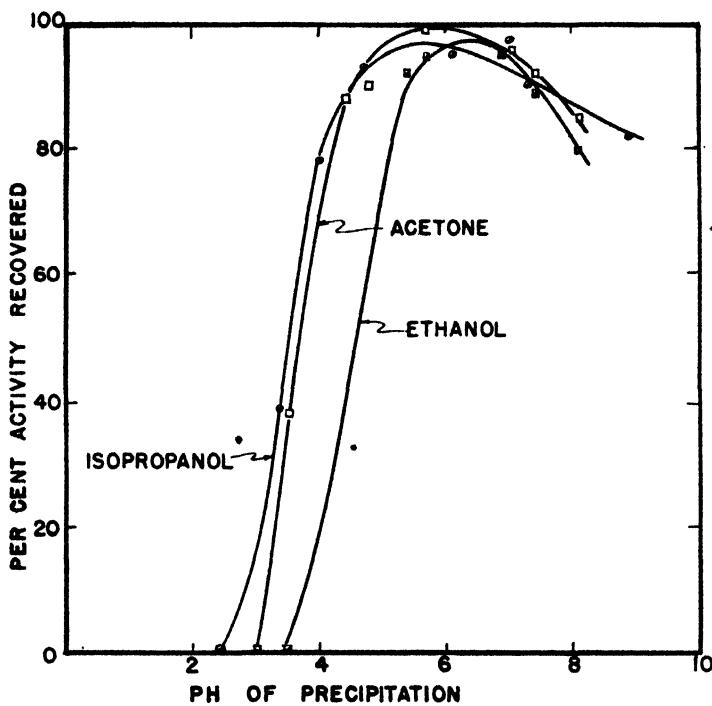


Fig. 3. Effect of hydrogen-ion concentration on recovery of amylase activity from mold bran extract.

The data shown by the curves of Fig. 3 were all obtained using distilled water extracts of mold bran. Precipitations with isopropanol were also carried out over a wide pH range, using a 0.2 per cent calcium chloride extract. The same pH optimum was found as for a distilled water extract, i.e., pH 5.5 to 6.0. However, a notable difference was observed in the rate of settling of the precipitates. In this case, as with ethanol, precipitates formed on the acid side of the optimum tended to remain in suspension while those formed near neutrality settled rapidly.

Influence of Quality and Quantity of Ions Present. There are two kinds of salt addition which might be made in an enzyme precipitation study. Certain techniques depend solely on the salt to precipitate the enzyme-active principal. Other methods incorporate a small amount of some salt as an aid to help carry down the active principal when an organic compound is used as the precipitating agent. Precipitates from distilled water extracts without salt addition were not easily filtered and were somewhat difficult to handle. The study of the effect of salts on precipitation was undertaken to find an aid in improving the physical characteristics of the precipitate, i.e., rate of flocculation and settling, filterability, and ease of powdering, without causing any decrease in activity of enzyme recovered.

TABLE IV
PRECIPITATION OF AMYLASE IN THE PRESENCE OF LOW
CONCENTRATION OF VARIOUS SALTS
(Salt concentrations: with isopropanol, 0.04 *N*,
with ethanol and acetone, 0.10 *N*)¹

Salt	Per cent amylase recovered		
	70% ethanol	60% isopropanol	60% acetone
NaCl	97	89	100
KCl	97	89	—
BaCl ₂	83	87	82
CaCl ₂	73	84	83
K ₂ SO ₄	—	—	95
(NH ₄) ₂ SO ₄	—	96	—
Na ₂ SO ₄	98	—	93
KH ₂ PO ₄	—	91	—
K ₂ HPO ₄	—	95	—
Na ₂ HPO ₄ · 12H ₂ O	93	—	93
NaH ₂ PO ₄ · H ₂ O	93	—	—
MgSO ₄ · 7H ₂ O	93	—	93
MgCl ₂ · 6H ₂ O	—	—	95
Al ₂ (SO ₄) ₃ · K ₂ SO ₄ · 24H ₂ O	—	0	—
Fe ₂ (SO ₄) ₃ · (NH ₄) ₂ SO ₄ · 24H ₂ O	—	0	—

¹ The addition of certain salts might influence enzyme recovery by producing changes in hydrogen ion concentration. However, this is believed unimportant because of the strong buffering action present in the mold bran extract and the low concentration of salts used. Also aluminum and iron are known to have inactivation potentialities.

The effect of various salts on precipitation by ethanol, isopropanol, and acetone was investigated, in each instance the salt being added before the precipitant to give 0.1 *N* concentration for ethanol and acetone and 0.04 *N* for isopropanol. The data are given in Table IV. It will be noted that the same salts were not used with all precipitants.

With the exception of calcium chloride and barium chloride, the salts studied with ethanol precipitation had little influence on the

recovery of enzyme. However, a pronounced influence on the physical characteristics was apparent: the phosphates tended to result in gummy precipitates; calcium and barium chloride gave flocculent, rapidly settling precipitates; and the other salts gave precipitates that settled rapidly but incompletely, leaving a cloudy supernatant liquid. The excellent physical characteristics of the precipitate formed in the presence of either calcium or barium chloride was a desirable feature but was nullified by the loss of enzyme activity.

In the isopropanol precipitation studies on the effect of different ions, several common salts were chosen to include representatives of mono-, di-, and trivalent cations and anions. The data of Table IV show that with an increase in the valence of the cation the activity of the precipitate decreased. The ferric and aluminum salts produced a voluminous precipitate which had no activity. Calcium and barium produced a lesser amount of precipitate. The monovalent cations produced the least precipitate but with the greatest activity. Again the separation obtained in the presence of the divalent cations seemed to be preferable and more complete than that obtained with the monovalent cations.

As far as the anions are concerned, the grouping by valence was not so distinct. Based on the activity of the precipitates produced from extracts, an anion series may be arranged as follows: phosphate, acetate, sulfate, chloride, nitrate, oxalate, citrate, tartrate, and sulfite. A variation of about 20% in enzyme activity existed between the first and last of this series.

The results with acetone, Table IV, support evidence gained from studies with the other precipitants showing that calcium and barium ions have a detrimental effect on the enzyme recovery. Further, the sulfate salts had a very pronounced effect on the physical characteristics of the acetone-produced precipitates from mold bran extracts. With magnesium sulfate in a concentration of 0.10 *N* the precipitate appeared as a semiliquid, sticky mass after centrifugation. If disodium phosphate was added to the aliquot containing magnesium sulfate, the precipitate appeared as a white floc that settled rather rapidly, leaving a clear supernatant liquid. In the tubes containing solutions of calcium and barium ions the precipitates had much the same appearance as those which contained the magnesium sulfate and disodium phosphate.

A brief study was made of the effect of concentration of calcium chloride and sodium chloride, when added to distilled water extracts, on the activity of the precipitates from 70% ethanol. The results are given in Fig. 4 and indicate a very detrimental effect of the calcium ion as compared to the sodium ion.

Additional information was obtained on the effect of calcium chloride concentration by a comparison of the tolerance of the three precipitating agents, ethanol, isopropanol, and acetone, to the presence of this salt. Aliquots of extract were adjusted to 0.10 and 0.20 *N*

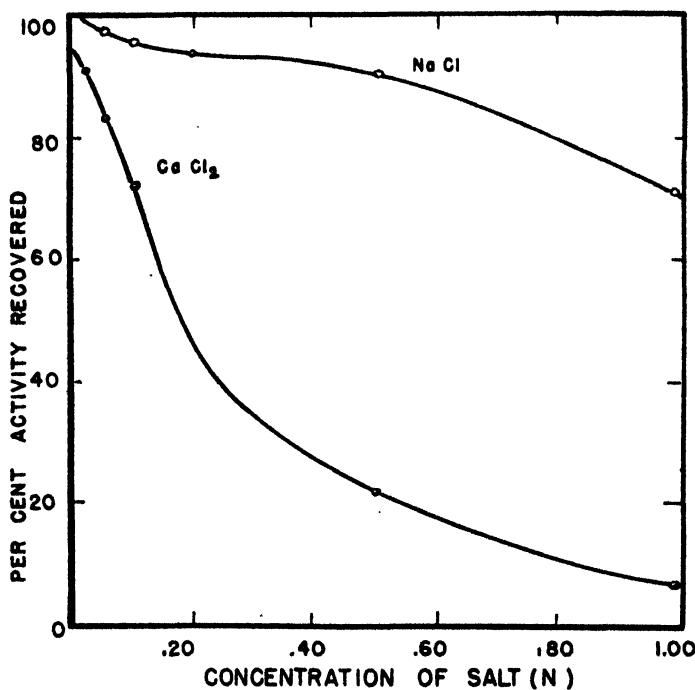


Fig. 4. Effect of salt concentration contrasting NaCl and CaCl₂ on the recovery of amylose activity from mold bran extract.

calcium chloride before being precipitated with 70% ethanol or 60% isopropanol or acetone. As shown in Fig. 5, when the activities of the precipitates thus formed were determined, it became evident that isopropanol precipitation was less influenced by the presence of excess calcium ions than precipitations by either of the other agents.

To isolate the individual salt effects, aliquots of an extract were dialyzed against various media and their behavior determined after dialysis. Four aliquots of extract were dialyzed respectively against distilled water, 0.043 *N* sodium chloride, 0.060 *N* calcium chloride, and 0.060 *N* disodium phosphate solutions for 48 hours at 10°C. After dialysis the extracts were adjusted to their original volumes and the activity of each was compared with that of an original extract. No loss of activity was observed in any of the solutions, or in a control allowed to stand at the same temperature without dialysis.

Aliquots of each of these dialyzed solutions were precipitated with 70% ethanol. For comparison, aliquots of an undialyzed extract, to which had been added equal amounts of salt, were precipitated under the same conditions. The results are given in Table V and demon-

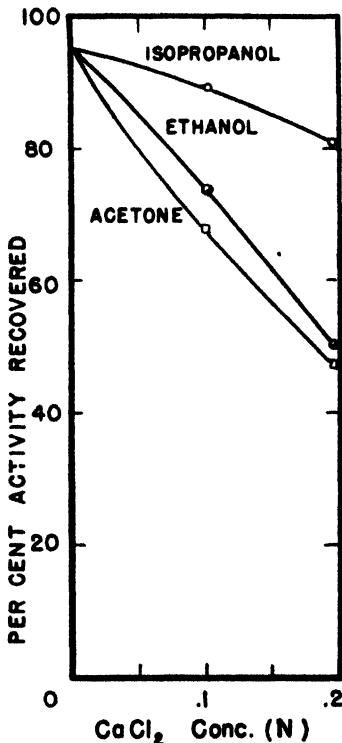


Fig. 5. Effect of calcium ion concentration on recovery of amylase activity.

strate that the presence of some salt is imperative to the recovery of enzyme activity in the precipitate. However, none of the salts used in the dialyzing solutions gave recoveries comparable to that obtained with the undialyzed extracts. The extracts dialyzed against distilled water produced only milkiness when ethanol was added, and when these were centrifuged very little precipitate settled out. The other dialyzed solutions produced small amounts of precipitate with cloudy supernatant liquids, indicating incomplete separation of the precipitated phase.

The foregoing results indicated that there was some dialyzable substance extracted from the bran, which served as an aid in recovering an active precipitate. To determine the source of this precipitating aid, i.e., whether it is in the natural wheat bran or is something formed or

added in the production of the mold bran, a sample of mold bran and a sample of ordinary wheat bran were extracted, filtered, and precipitated under the same conditions. The wheat bran extract gave a precipitate with physical characteristics similar in appearance to those of the precipitate from mold bran.

TABLE V
PRECIPITATION OF AMYLASE BY 70% ETHANOL FROM MOLD BRAN EXTRACTS
DIALYZED AGAINST DISTILLED WATER AND AGAINST SALT SOLUTIONS

Dialysis medium	Salt concentration (N)	Per cent amylase recovered	
		Dialyzed solution	Undialyzed solution
Distilled water	—	21	100
NaCl	0.043	89	100
CaCl ₂	0.060	89	83
Na ₂ HPO ₄ ·12H ₂ O	0.060	59	100

An analysis of wheat bran reported by Bailey (1) indicates a high content of the four elements, phosphorus, magnesium, calcium, and potassium. These elements were considered singly and in combination to determine their effect on the appearance of the precipitate.

A quantity of mold bran extract was dialyzed for 24 hours against distilled water. Aliquots of this extract were adjusted to various concentrations with various salts and precipitated with 70% ethanol. Any one of the salts studied aided in recovery of the enzyme. However, as may be seen in Table VI, some appeared to cause an increasing loss of enzyme with increases in concentration. Only the magnesium

TABLE VI
RECOVERY OF AMYLASE BY ETHANOL PRECIPITATION IN DIALYZED
MOLD BRAN EXTRACTS WITH ADDED SALTS

Salt added	Per cent amylase recovered	
	0.10 N salt	0.50 N salt
No salt added	less than 5	—
NaCl	97	97
KCl	93	93
MgCl ₂ ·6H ₂ O	90	75
CaCl ₂	52	less than 5
K ₂ HPO ₄	13	30
0.012 N salt		
CaCl ₂	69	
Na ₂ HPO ₄ ·12H ₂ O	69	
0.023 N salt		
MgCl ₂ ·6H ₂ O	94	98
Na ₂ HPO ₄ ·12H ₂ O	94	96
0.036 N salt		

chloride-disodium phosphate combination produced a white, flocculent precipitate characteristic of undialyzed solutions. All other salts or combinations produced milky, slow-settling precipitates.

In an investigation to determine the optimum concentration of magnesium sulfate and disodium phosphate required for precipitation, no sharp crest occurred. However, concentrations of 0.028 *N* and greater left increasing amounts of insoluble residue when the precipitates were redissolved. Magnesium sulfate was substituted for magnesium chloride because of its greater ease of handling and because no difference was found in the effectiveness of the two salts as precipitating aids with ethanol employed as the precipitant. Variations in the concentration of magnesium sulfate and disodium phosphate from 0.019 *N* to 0.046 *N* had no effect on the per cent recovery of enzyme.

Studies were conducted to determine the effect of varying ratios of the magnesium ion and monohydrogen phosphate ion on the activity of the precipitate, and the data obtained are given in Table VII. An

TABLE VII

EFFECT OF VARIABLE $(Mg)^{++}/(HPO_4)^{--}$ RATIO AND TIME OF STANDING ON THE AMYLASE ACTIVITY OF THE PRECIPITATE PRODUCED WITH 70% (BY VOLUME) ETHANOL

Concentration (<i>N</i>) $MgSO_4 \cdot 7H_2O$	Concentration (<i>N</i>) $Na_2HPO_4 \cdot 12H_2O$	$(Mg)^{++}/(HPO_4)^{--}$ ratio	Per cent amylase recovered	
			10 min.	3 hrs.
0.047	0.019	2.6	90	62
0.038	0.019	2.0	—	72
0.029	0.019	1.5	90	78
0.019	0.019	1.0	—	87
0.019	0.029	0.66	98	92
0.019	0.038	0.50	—	92
0.019	0.056	0.34	98	92
0.019	0.073	0.26	—	96

excess of magnesium was detrimental to the activity of the precipitate. This fact supports evidence of loss caused by excess of the divalent calcium and barium ions when salts of these ions were studied as precipitation aids. When the element of time was introduced along with the factor of ionic ratio, it was demonstrated that an excess of monohydrogen phosphate ion stabilized the precipitate. One series of precipitations was made and allowed to stand for 3 hours before being centrifuged, while another was centrifuged immediately after precipitation. With an increase in the $(Mg)^{++}/(HPO_4)^{--}$ ratio from 0.34 to 1.0 the rate of settling increased and the clarity of the supernatant liquid decreased. Ratios below 0.34 produced a cloudy supernatant liquid and rapid settling.

The tendency of acetone to produce discolored, gummy, and water-insoluble precipitates removed this precipitant from those considered for additional investigation.

Batch Precipitation. Having established that ethanol and isopropanol were the more promising precipitating agents, an effort was made to prepare a quantity of the enzyme concentrate with each of these precipitants. Attempts to dry precipitates from ethanol were rewarded with isolates of high activity and good physical characteristics except for water solubility. By using a concentration of 0.25% magnesium sulfate and 0.72% disodium phosphate, by weight, in the enzyme extract, a white to buff precipitate was formed that had 10 times the activity of the original mold bran. The precipitate was separated by centrifugation, resuspended in 95% ethanol, and filtered. Approximately 68% yield was obtained, based on the total enzyme content of the mold bran.

In the batch precipitations with isopropanol similar results were obtained. In Table VIII are listed the data on four trials at isolating and drying concentrates from isopropanol precipitation.

TABLE VIII
RECOVERY OF AMYLASE IN PRECIPITATES FORMED WITH ISOPROPANOL

Batch	Volume of extract	Precipitate activity <i>dT</i> of 0.005 g. ¹	Yield recovery			
			ml.	min.	g.	%
1	100	40.5		1.94		92
2	100	9.0		0.39		71
3	100	13.0		0.68		86
4	1000	10.5		6.45		74

¹ "dT" signifies dextrinization time.

In each case 0.25% magnesium sulfate and 0.50% disodium phosphate, by weight, were added to the extract before precipitation with 60% isopropanol. The residue after centrifugation was dispersed in a volume of isopropanol one-fifth the original volume of the extract.

The precipitates formed were separated principally by centrifugation since their physical properties did not permit easy filtration either by gravity or suction. Either a supercentrifuge or large capacity centrifuge was found necessary to obtain satisfactory separation.

Properties of Precipitates. When comparing the activities of the two types of precipitates, one from ethanol and the other from isopropanol, it was found that the one resulting from isopropanol was somewhat more active. For example, 0.05 g. of the mold bran gave a dextrinizing time of 13 minutes; 0.005 g. of the ethanol precipitate gave an activity represented by the same dextrinizing time, 13.0

minutes; and 0.005 g. of the isopropanol precipitate gave a dextrinizing activity of 10.5 minutes.

In evaluating and comparing the saccharifying power of mold bran and concentrates prepared from it, extracts of the above three sources were adjusted to the same dextrinizing activity and then equal amounts ($dT=20$ minutes) of the extracts added to a 2% starch substrate plus yeast nutrients and allowed to digest for 1 hour at 30°C. At the end of this hour 0.5 g. of compressed yeast was added to the fermentation cup and the fermentation was followed manometrically. Readings were taken at 1, 3, 5, 10, 21, and 22 hours. The data in Table IX

TABLE IX
SACCHARIFYING POWER OF AMYLASE EXTRACTS FROM VARIOUS SOURCES

Enzyme source	Wt. equal (mg.) $dT=20$ min.	Pressure—mm. mercury ¹					
		1 hr.	3 hrs.	5 hrs.	10 hrs.	21 hrs.	22 hrs.
FUNGAL CONCENTRATES							
Commercial A	1.3	17	81	147	251	327	336
Commercial B	1.0	15	80	138	270	323	329
Commercial C	0.76	17	67	144	276	331	338
Ethanol precipitate	3.5	14	59	123	272	339	341
Isopropanol precipitate	2.6	15	65	140	293	383	387
FUNGAL BRANS							
A	6.1	16	67	145	281	361	364
B	19.7	13	71	153	290	363	366
C	12.5	14	72	153	280	343	346
D	31.4	15	63	138	296	390	390

¹ These figures give an indication of the rate of fermentable sugar production under fermentation conditions.

include, for comparison, information on the other mold brans and fungal concentrates.

It will be noted that all of the enzyme sources listed in Table IX gave approximately the same fermentation pattern. However, the commercial mold bran D and the isopropanol precipitate gave greater total conversion than any other of the enzyme sources, as indicated by the readings at 22 hours. It also should be noted that the isopropanol precipitate was superior to the ethanol precipitate in total starch conversion.

Discussion

The data herewith presented show conclusively that an active precipitate may readily be obtained from mold bran extracts by the

use of a water-miscible organic compound. However, to recover high yields of enzyme in a precipitate qualitatively similar enzymatically to the mold bran and with desirable physical characteristics, the precipitating conditions should be adjusted with some care. To satisfy these conditions and, in addition, to give greatest enzyme recovery at lowest concentration of precipitating agent, isopropanol was the compound of choice among those tested. In addition isopropanol precipitation proved to have a low degree of sensitivity to the presence of otherwise undesirable salts.

The influence of certain ions on the recovery of enzyme and on the physical characteristics of the precipitate has considerable significance in commercial application. The presence of certain bivalent cations should be avoided. This applies particularly to the commonly occurring calcium ions. On the other hand, the presence of magnesium ions is desirable, but only if in conjunction with balancing phosphate ions. In fact, the presence of adequate magnesium and phosphate ions appeared to be a prerequisite to satisfactory precipitation. Customarily, these should be present in amounts supplementary to the quantities already present in the mold bran extract.

Since adequate precipitation could not be obtained with extracts dialyzed free of salts, it appears that the problems of precipitation are mainly those related to these salts. Satisfactory precipitation with a water-miscible organic compound proved to depend on the adjustment of conditions such that a good precipitate of magnesium phosphate was obtained, which in turn carried with it the desired enzyme in a concentrated form.

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HYDROLYSIS OF THE AMYLOPECTINS FROM VARIOUS STARCHES WITH BETA-AMYLASE¹

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ABSTRACT

The amylopectin or branched-chain fractions of corn, wheat, white potato, sweet potato, and tapioca starches were isolated by removal of the amylose or linear fraction after its precipitation with *n*-butanol, then degraded by beta-amylase to the limit dextrans. The extent of conversion to maltose, yields of limit dextrans and crystalline maltose hydrate, phosphorus contents, alkali labilities, iodine sorptions, specific optical rotations, and properties of the triacetyl derivatives were determined.

The branched-chain fractions from the different starches were alike in extent of conversion by beta-amylase, alkali lability, specific optical rotation, and in some properties of the acetates. They differed in phosphorus content, the nature of the phosphorus present, and iodine sorption. The root and tuber limit dextrans retained phosphorus, whereas the cereal limit dextrans did not. Evidence was found which was interpreted as indicating the existence in some starches, particularly corn and sweet potato, of a fraction intermediate in the extent of branching between linear amylose and the average for branched amylopectin.

References are made in recent literature to the uncertainty that exists as to whether the amylopectins of starches from different plant

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sources are alike or different, and the need for further investigations on the structure of amylopectin by means of enzymic degradation has been indicated.

In studies of the structure of the amylopectin or branched-chain fraction of starch by enzymic degradation, it is desirable that the amylopectin first be isolated. Much of the work in the past has been performed on whole starch, usually degraded soluble starch (5, 7, 8, 9, 13). In other studies (4, 14, 15, 17, 18, 23) attempts were made to isolate the amylopectin fraction, but the methods of fractionation used (hot water extraction and electrodialysis) generally have yielded amylopectins of doubtful purity. In recent years Schoch (24) has developed a more efficient method for separating the linear from the non-linear molecules of starch, involving the crystallization of the linear fraction as a complex with *n*-butanol; but only Kerr (11) has applied the method to isolate branched material for degradation with beta-amylase. Kerr's work was confined to branched-chain fractions from corn starch. The waxy starches occur in nature virtually free from linear molecules. Their degradation by beta-amylase also has been studied (3, 16, 17, 19).

The work reported here compares the amylopectin fractions of corn, wheat, white potato, sweet potato, and tapioca starches by determining the extent of their conversion to maltose by beta-amylase. The amylopectins and corresponding limit dextrans were examined for alkali lability, specific optical rotation, properties of the acetate derivatives, phosphorus content, iodine sorption, and anomalous linkage content as shown by the degradation of the limit dextrin triacetates in hydrogen bromide-acetyl bromide-acetic acid reagent (10). The starches were not autoclaved before fractionation, the amylopectins were isolated by precipitation of the amylose fractions as crystalline complexes with *n*-butanol, and the preparation of beta-amylase from ungerminated wheat was free from alpha-amylase and maltase.

Materials and Methods

Preparation of Amylopectins. Most of the starches selected were isolated in this laboratory under conditions which gave products of high purity with a minimum degree of alteration from the native state of the granules. Only distilled water was used for steeping, except for the wheat which required a 0.1% sulfur dioxide steep. The tapioca, corn II, and sweet potato I starches were high-grade commercial samples. The corn and wheat starches were defatted by methanol extraction in a Soxhlet apparatus, whereas the root and tuber starches contained 0.2% or less of methanol-extractable substances at the outset and were not defatted.

The method of Schoch (24) for fractionating starch with *n*-butanol has been altered in this laboratory by Karjala, Wolff, and Olds (unpublished experiments) to avoid degrading the starch by autoclaving. Starch was pretreated with liquid ammonia at -35°C. to swell and partially disorganize the granular structure. Mixing ethanol with the suspension of starch in liquid ammonia allowed isolation of the starch, after evaporation of the ammonia, in a granular state. The dried starch was reactive toward acetylation, showed no crosses by polarized light, and could be dispersed effectively in water saturated with *n*-butanol at 90°C. Following Schoch's method the linear fraction was crystallized in Dewar vessels and removed from the branched material by the continuous supercentrifuge. One additional pass through the supercentrifuge produced a homogeneous amylopectin solution (2-3% concentration, pH 6.5 ± 0.2). After distillation *in vacuo* below 50°C. a nearly clear, butanol-free paste remained, 8-10% amylopectin by weight. The concentration was determined by drying weighed samples of paste to constant weight under an infra-red lamp at 120°C.

The major part of this paste was used for hydrolysis by beta-amylase; the remainder was precipitated in nine parts of ethanol in a Waring Blender to obtain a fluffy powder easily soluble in water. White potato starch II was dispersed for fractionation without pre-treatment with liquid ammonia by simply adding a suspension of it in butanol to stirred butanol-water at 90°C. The yields, 66% for corn, 72-75% for white potato, sweet potato, and tapioca amylopectins, were close to the amounts expected from the reported amylose contents of these starches (1, 24), considering that 3-6% of butanol-water soluble substance in the starch was precipitated with the amylose fraction.

In addition to the amylopectins isolated as described above, a fraction of the amylopectin of corn starch II was separated from the crude amylose-butanol precipitate. The crude amylose complex (approximately 100 g. dry weight) was dissolved in 9 liters of water saturated with *n*-butanol at 90°C. and autoclaved for 1 hour at pH 6.7 (initial) to 6.3 (final). After 2 days of slow cooling with stirring, the recrystallized amylose-butanol complex was removed cleanly by supercentrifugation, yielding 80 g. of purified amylose (iodine sorption: 194 mg./g.). The centrifugate was concentrated *in vacuo* to a 5% paste. A part of the paste was precipitated in ethanol and the remainder was hydrolyzed with beta-amylase.

Preparation of Beta-Amylase. The method was essentially that of Van Klinkenberg (29) as applied by Hanes (6) to barley and Haworth *et al.* (8) to wheat. The continuous supercentrifuge was used to separate the 50% (by volume) cold aqueous ethanol-soluble, 80% cold

aqueous ethanol-insoluble fraction from finely ground whole wheat (Trumbull variety, soft red winter wheat; grown at Wooster, Ohio, 1944 crop). The dry powder obtained (35 g. from 3 kg. wheat) was extracted with 2 liters of water in portions to form a solution of the enzyme. The filtered, unbuffered solution, preserved with toluene and thymol and kept below 5°C., was used as a source of beta-amylase for all the hydrolyses. Filtering before each use removed an amorphous precipitate which formed in the solution on standing. The pH of the solution increased from 6.0 to 6.7 in the first 6 weeks but remained constant thereafter. Seven months after preparation, at the end of the experiments, the strength per gram of the solution was 1.15 Kneen-Sandstedt beta-amylase units (12).

The beta-amylase preparation was free from alpha-amylase because the percentage of amylopectin hydrolyzed in the presence of a large excess of beta-amylase reached a limit that was constant after 24, 30, 48, and 60 hours of incubation at 36°C., pH 6.5. The method of Olson, Evans, and Dickson (20) for detecting alpha-amylase in beta-amylase preparations gave negative results on other solutions prepared in identical manner from the same wheat. Maltase was absent because the optical rotation and reducing power of a solution of maltose and the enzyme were constant. The reducing power of the enzyme solution alone toward Fehling's solution was 0.6 mg. maltose equivalent per gram initially, and decreased with aging.

Hydrolysis of Amylopectins with Beta-Amylase. To facilitate the quantitative isolation of limit dextrin and maltose hydrate, no buffer salts were added to the substrate. The pH remained constant in the range 6.4–6.9 throughout all the hydrolyses.

Example: One kg. of unbuffered paste containing 95.0 g. of amylopectin was treated with 200 g. of the enzyme solution. After mixing 2–3 ml. of toluene into the paste, the flask was immersed in a constant temperature bath at 36°C. and shaken at intervals. Weighed samples were taken for reducing sugar determinations at 6, 18, and 24 hours. After 24 hours, 25 g. additional enzyme solution was added, and the flask was kept at 36°C. for another day. At 30 and 48 hours the reducing power was again determined; in no case did the conversion continue after the second addition of enzyme. The rate of hydrolysis in a typical case (wheat amylopectin), as measured by the reducing action of the maltose formed, follows: In 2, 6, 24, 30, and 48 hours at 36°C. (C 7.9%, pH 6.7), 51.0, 55.0, 55.8, 55.4, and 55.5%, respectively, of the amylopectin was found converted to maltose.

A duplicate series of hydrolyses was run at nearly the same concentrations, but on a smaller scale, by dissolving the precipitated amylopectins (20 g.) in water (180 g.) at 80°C., diluting to 9% concentration,

cooling, and adding 30 g. enzyme solution. The extents of hydrolysis and recoveries of limit dextrin and maltose were virtually the same as obtained in the first series.

Isolation of Limit Dextrins and Maltose Hydrate. The limit dextrins were precipitated from the hydrolyzates by adding ethanol to 60% concentration by volume. To obtain complete precipitation of the potato dextrins, approximately 1 g. of sodium chloride was added. The gummy mass of dextrin was squeezed, redissolved in 10-12 parts water, and precipitated in the Waring Blender in the ratio one part paste to nine parts absolute ethanol. By frequently rinsing down the sides of the Blender, the precipitating jars, and the Buchner funnel with ethanol, no significant losses occurred. The dextrin was dried in a vacuum desiccator, then at 80°C. in the vacuum oven. As the dextrin was weighed to determine the yield, a sample was taken for a moisture determination. The limit dextrin was purified by redissolving in hot water, precipitating twice at 50% ethanol concentration and a third time in absolute ethanol as before. The reducing powers of the purified dextrins were approximately 0.1% maltose equivalent; the nitrogen contents were 0.05-0.07% compared to 0.01-0.02% for the amylopectins.

For isolation of the maltose hydrate, the alcoholic liquors from the first and second precipitations were filtered, concentrated *in vacuo* below 40°C., refiltered, and further concentrated to a syrup. Crystallization was effected at 65 to 75% ethanol concentration with seeding. The mother liquors were concentrated and two additional crops of crystals were obtained. The yields of maltose hydrate were calculated from the maltose content of the crystalline fractions as determined by their optical rotation, on the assumption that the impurities were optically inactive. The purities of the first crops were usually higher than 98%. Highly purified beta-maltose hydrate was prepared for standardization of the reducing sugar method by recrystallizing three times from 60-65% ethanol and drying over calcium chloride in a vacuum desiccator. The product was finally dried at 50°C., 1 mm. pressure, for 5 hours in a vacuum oven. $[\alpha]_D^{25}$ after 5 minutes in solution, + 114°; final, + 130.4° (C 4.0, H₂O). "Melting" range: 121°-125°C. (varying within these limits with degree of subdivision and rate of heating); the opaque "melt" frothed at 140°-150°C. and was then clear and colorless. Water content: 5.01%; calculated for the monohydrate 5.00%. On exposure at 40-50% relative humidity, the water content increased to 5.10 ± 0.03%. Water was determined by drying 1-g. samples to constant weight in an Abderhalden apparatus over boiling toluene (pressure less than 1 mm.); the results were confirmed by Karl Fisher reagent.

Acetylation of Limit Dextrans. Five g. of the purified limit dextrin were mechanically stirred with 50 ml. dry pyridine in a flask protected from atmospheric moisture. A mixture of 30 ml. acetic anhydride (three times theory) in 20 ml. pyridine was added, and the mixture was stirred 6 hours in a bath at 100°C. A double quantity of the acetic anhydride-pyridine mixture was necessary in the acetylation of the potato limit dextrans because they formed stiff gels that were difficult to stir. The limit dextrin acetate was precipitated in ethanol using the Waring Blender, washed thoroughly in 50% aqueous ethanol, and dried *in vacuo* at 25°C. and 100°C. This acetylation procedure was essentially that of Whistler, Jeanes, and Hilbert (31). The acetyl contents were $44.8 \pm 0.3\%$ for all but sweet potato I acetate which was 44.2% acetyl; the theoretical amount for complete acetylation is 44.8%.

Analytical Methods. The maltose in the hydrolyzates was determined by the Munson-Walker oxidation with Fehling's solution. The reduced copper was determined without filtration by the iodometric "cuprous" titration of Shaffer and Hartmann (27). Samples of hydrolyzate were taken to contain approximately 100 mg. maltose hydrate. Walker's table for maltose hydrate (30) was found to give results correct within 1.5% (average deviation $\pm 0.7\%$) when 100 mg. of the purified maltose hydrate was used as a standard. The reducing power contributed by the added enzyme solution was less than the probable error of the maltose determination.

The analytical procedure for phosphorus was that of Truog and Meyer (28). Alkali lability was determined by the method of Schoch and Jensen (25), except that the bottles were heated in steam baths. To determine iodine sorption, 40 mg. of the starch fraction in 100 ml. solution (0.05 N with potassium iodide and potassium chloride) was titrated with 0.001 N iodine (1, 32). From a graph of the titration, the point of inflection was determined. By subtracting from the volume of iodine solution required to reach the point of inflection that volume used in the blank titration to reach the same EMF as the point of inflection, the amount of iodine sorbed by each starch fraction was estimated. No distinction was made between the iodine actually in complex formation and that sorbed mechanically. The optical rotations of the limit dextrin acetates in the hydrogen bromide-acetic acid-acetyl bromide reagent were determined exactly as prescribed by Jeanes and Hilbert (10).

Results³ and Discussion

The average results for the hydrolyses are given in Table I. The percentages of amylopectin hydrolyzed to maltose hydrate (column 2)

³ All numerical results in this paper are calculated to the dry weight of the starch fractions.

were calculated from the constant values of the reducing powers of the hydrolyzates. The sums of columns 2 and 3 in all cases fall within $100 \pm 1\%$.

TABLE I

HYDROLYSIS OF AMYLOPECTINS WITH BETA-AMYLASE—CONCENTRATION OF AMYLOPECTIN 6-8%, pH 6.4-6.9, TEMPERATURE 36°C., TIME 48 HOURS

Source of amylopectin	Hydrolysis to maltose hydrate (by copper reduction)	Limit dextrin isolated (dry basis)	Maltose hydrate isolated
	(2)	(3)	(4)
	% of theory	%	% ¹
Corn I	57	43.5	96
Corn II ²	56.5	44.5	—
Wheat	55.5	45	91
Sweet potato I	54	45	94
Sweet potato II	54	46	—
White potato I	54	47	95
White potato II	53.5	46	91
White potato IIa ³	53.5	47	—
Tapioca	53	47.5	95

¹ Per cent of the amount expected, calculated from the limit dextrin isolated.

² Corn amylopectin II was isolated from crude amylose-butanol precipitate by recrystallizing the amylose and recovering the solubles.

³ Potato starch IIa was not treated with liquid ammonia before fractionation, otherwise the same as II.

The root and tuber amylopectins (tapioca, sweet potato, and white potato) were hydrolyzed by beta-amylase to the same extent, 53-54%; the cereal amylopectins (wheat and corn) were hydrolyzed to a slightly greater extent, 55-57%. However, the iodine sorption numbers of the cereal amylopectins were relatively high and decreased appreciably after the action of beta-amylase (Table IV), suggesting the contamination of the cereal amylopectins with linear or nearly linear molecules. If a correction is made on the assumption that the difference between the iodine titers of the amylopectin and limit dextrin is a measure of the contamination with linear molecules of amylose, the corrected percentages of hydrolysis for the wheat and corn amylopectins would be 54 and 55%, respectively. Then the amylopectins from various plant sources would have approximately the same limit of conversion by beta-amylase.

The results of the alkali lability determinations in Table II show no significant differences among the amylopectins and limit dextrans. The alkali numbers of the limit dextrans are approximately double those of the amylopectins, lending support to the view (11, 26) that the alkali lability of a starch fraction is dependent on the proportion of reducing end groups and varies inversely with the molecular weight if the linear branches containing the reducing end groups are of the same length.

The specific optical rotations of the amylopectins and limit dextrans were determined at 1% concentration in (a) water, (b) 33% aqueous calcium chloride, and (c) 5% aqueous sodium hydroxide. The specific rotations for all varieties were the same ($\pm 1^\circ$) in each solvent. For the amylopectins they were $[\alpha]_D^{25}$ (a) +198°, (b) +204°, (c) +162°; and for the limit dextrans (a) +197°, (b) +201°, (c) +162°. These values are much higher than those obtained by Haworth and co-workers (8) for the beta-amylase limit dextrin of soluble starch.

TABLE II
ALKALI LABILITY NUMBERS OF AMYLOPECTINS AND LIMIT DEXTRINS

Source of amylopectin	Amylopectin	Limit dextrin	Ratio
Corn I	2.5	5.2	2.1
Corn II ¹	5.7	5.1	—
Wheat	2.9	4.3	1.5
Sweet potato I	2.6	4.6	1.8
White potato I	1.9	3.2	1.7
White potato II	1.4	4.1	2.9
Tapioca	1.5	4.1	2.7

¹ Corn amylopectin II was isolated from crude amylose-butanol precipitate by recrystallizing the amylose and recovering the solubles.

The triacetates of the limit dextrans from corn, wheat, and tapioca amylopectins were soluble in chloroform, $[\alpha]_D^{25}$ + 165°, + 165°, + 166° (C 1.0) respectively; those of the white potato and sweet potato limit dextrans formed swollen, gelatinous masses that would not disperse. The white potato and sweet potato acetates contained higher percentages of phosphorus than the soluble acetates. Each limit dextrin acetate sintered in capillary tubes in the range 170°–190°C. and softened slowly thereafter up to 250°C.; however, the corn, wheat, and tapioca acetates became clear in the tubes at about 225°C., while the white potato and sweet potato acetates did not.

The end optical rotations of the triacetates of all the limit dextrans in the hydrogen bromide-acetic acid-acetyl bromide reagent of Jeanes and Hilbert (10) fell within the narrow range $[\alpha]_D^{25}$ + 15.8 \pm 0.1° S. Since the experimental error was approximately 0.05° S, the different varieties of limit dextrans have the same rotation in the Jeanes-Hilbert reagent; hence, there is probably no great variation in the extent of branching among the different varieties of amylopectin.

The phosphorus contents of the amylopectins and limit dextrans are given in Table III. For the root and tuber starches the phosphorus contents of the limit dextrans are approximately double those of the amylopectins. This indicates that the phosphorus present in the potato, sweet potato, and tapioca starches is chemically bound in the half of the molecule remaining as limit dextrin. The small amount

of phosphorus in the corn and wheat amylopectins is not an integral part of the residual dextrin because the degradation of the cereal amylopectins resulted in a decrease in phosphorus content. These results are in accord with those of Posternak (21) and others (2, 22). Since

TABLE III
PHOSPHORUS CONTENTS OF AMYLOPECTINS AND LIMIT DEXTRINS

Source of amylopectin	Phosphorus		Ratio
	Amylopectin	Limit dextrin	
	%	%	
Corn	0.009	0.005	0.6
Wheat	0.007	0.006	0.9
Sweet potato I	0.017	0.035	2.1
Sweet potato II	0.019	0.032	1.7
White potato I	0.093	0.182	2.0
White potato II	0.089	0.161	1.8
White potato IIa ¹	0.102	0.174	1.7
Tapioca	0.009	0.016	1.8

¹ Potato starch IIa was not treated with liquid ammonia before fractionation, otherwise the same as II.

Posternak showed white potato, arrowroot, and sago starches contain chemically bound phosphorus and the present work extends the list to include sweet potato and tapioca starches, it is probable that all root and stem starches contain bound phosphorus and only the cereal grains give starches with no significant amount of phosphorus in combination with the glucosidic units. The fact that the conversion limits of the corn and wheat amylopectins are slightly higher than those of the root and tuber amylopectins has been attributed to the presence of amylose impurity in the cereal amylopectins; on the other hand, the blocking of enzymic action at glucose units containing phosphorus would cause a similar result.

The iodine sorption values, expressed as milligrams iodine sorbed per gram of starch fraction, are listed in Table IV. Tapioca and white potato amylopectins and limit dextrins showed minimal sorptions of iodine. Corn and wheat amylopectins sorbed appreciable quantities of iodine, but in producing the limit dextrins much of the iodine-sorbing material was lost, probably because amylose was present as an impurity. The hydrolysis of sweet potato amylopectins to limit dextrins gave a different result. The two amylopectins sorbed about the same amount of iodine as corn amylopectin, but the limit dextrins retained more of the iodine-sorbing factor. A similar effect was obtained in corn amylopectin II that was isolated from the amylose fraction; the limit dextrin sorbed nearly as much iodine as the amylo-

pectin. This amylopectin may have contained a small amount of amylose as indicated by the higher alkali number in Table II; but the alkali number of the limit dextrin was lower and normal, showing that the alkali-labile impurity was removed by beta-amylase.

TABLE IV

IODINE SORPTIONS OF AMYLOPECTINS AND LIMIT DEXTRINS—CONCENTRATION 40 MG. PER 100 ML., TEMPERATURE 24°–26°C.

Source of amylopectin	Amylopectin	Limit dextrin
	mg./g.	mg./g.
Tapioca	3.0	1.9
White potato I	3.7	3.4
White potato II	4.2	3.4
Wheat	8.6	3.5
Corn I	15.3	7.7
Corn II ¹	18.1	16.7
Sweet potato I	16.0	12.4
Sweet potato II	16.3	11.8

¹ Corn amylopectin II was isolated from crude amylose-butanol precipitate by recrystallizing the amylose and recovering the solubles.

Since iodine-sorbing material is found in the limit dextrans after the completed action of beta-amylase, the presence of some molecules with a low degree of branching and /or relatively long, straight trunks is indicated. Two methods capable of removing linear amylose from branched amylopectin, adsorption on cotton and precipitation with *n*-butanol, were successful in removing a fraction (5–8%) from the limit dextrin of sweet potato I amylopectin. The butanol precipitate was not crystalline and sorbed much less iodine (36 mg./g.) than amylose (200 mg./g.), but much more than any amylopectin or limit dextrin (1–16 mg./g.). These phenomena are being investigated further to determine whether some amylopectins, especially those of sweet potato and corn, contain molecules intermediate in the extent of branching between amylose and the average for amylopectin.

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**STUDIES ON EXPERIMENTAL BAKING TESTS. IV.
COMBINED EFFECTS OF YEAST, SALT, AND
SUGAR ON GASSING RATES¹**

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ABSTRACT

A baking formula of 3% yeast, 1.75% salt, 5% sugar plus an ammonium salt was found necessary for adequate gassing up to and through the pan-proof period under the A.A.C.C. standard procedure.

Increased sucrose in the formula augmented the initial gassing rate and diminished and delayed the fermentation attributed to maltose. Salt exhibited an over-all depressing and extending effect on fermentation. An interaction of salt and sugar in fermenting doughs was observed. The ammonium ion was the effective ion in the acceleration of maltose fermentation by ammonium dihydrogen phosphate.

Increasing yeast concentrations resulted in acceleration of fermentation and earlier exhaustion of fermentable sugars.

Baking data obtained with a formula based on these studies showed a very close relationship between protein content and loaf volume, i.e., gassing power had been eliminated as a variable and the loaf volume was strictly dependent on flour strength.

Gassing power and gas retention have long been considered independent variables influencing the baking properties of wheat flour doughs. Gassing power to a large degree is dependent on the formula used, while optimum gas retention is an inherent property of the flour itself, depending to great extent, though not entirely, on the protein content. Recent work has indicated that certain factors, formerly regarded as influencing gassing power alone, exert an effect on the gas

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retention. Sandstedt, Jolitz, and Blish (13), in a study on synthetic flours, demonstrated that removal of the amyloextrin, or small granule portion of the starch, produces improved loaf volume. Kneen and Sandstedt (5) observed that improvement results from addition of alpha-amylase preparations to sugar-rich doughs from which presumably gas production had been removed as a factor. But despite this evidence of an interaction of those flour components responsible for gas production and gas retention, the gassing power of a dough must still be largely governed by simple additions to the baking formula.

Larmour and Brockington (8, 9), and Larmour and Bergsteinsson (7), investigating the effects on gassing rates of various fermentation procedures, dosages of bromate, various acidic, basic, and neutral salts, and a number of the common sugars, concluded that the effects of bromate and of punching are negligible; that bakers' yeast distinctly prefers sucrose, glucose, and fructose to maltose; that apart from the influence of pH exerted by the acidic and basic salts, the presence of salt retards yeast fermentation. They observed also that the gassing-rate curves with small amounts of added sugar always showed double maxima, a phenomenon interpreted as due to preferential fermentation of sucrose by the yeast.

The yeast used by Landis and Frey (6) in a methods study produced a double maxima gassing-rate curve. Eisenberg (4), with three different commercial bakers' yeasts, obtained three different types of gassing curves with flour doughs, one type of which showed the first maximum reduced to a mere inflection.

Blish and Sandstedt (3) postulated the existence of "Factor M," a biocatalytic activator, specifically effective in the fermentation of maltose. Sandstedt and Blish (11, 12) presented confirmatory evidence for the existence of this factor and recommended proofing doughs to constant height in laboratory test baking in order to minimize the effects of differing contents of "Factor M."

Schultz, Atkin, and Frey (14) attributed the "Factor M" effect to lack of amino nitrogen, but Ofelt and Sandstedt (10) failed to equalize proofing rates with additions of amino nitrogen, although they did so successfully with 0.5% dosages of ammonium dihydrogen phosphate. Larmour and Bergsteinsson (7) had studied the effects of ammonium salts and had noted the specific effect of the ammonium ion on the rate of fermentation of maltose in flour doughs.

Atkin, Schultz, and Frey (1) developed a nutrient solution capable of supporting the fermentation of sucrose at a rate equivalent to that of a flour substrate. Ammonium compounds are not included among the many constituents of this medium, but amino nitrogen is required. Many of the constituents are present in flour, some of them

in excess amounts. There may be a parallelism here, the active components of the nutrient solution being not necessarily the active factors in a flour substrate.

Clarification of the exact role of those baking constituents that have a known effect on the gassing power of a dough may permit development of baking formulas where the gassing power is independent of flour sources. The baking test, under such formulas, would evaluate flour strength only, unobscured by other factors. The more common dough ingredients having a known effect on gas production are yeast, sugar, salt, and certain yeast foods.

This is the report of the results of a study of the action and interaction of these baking constituents on the gas production of doughs. Using the data obtained, the degree of relationship between protein content and baking data is determined.

Materials and Methods

Gas production and gas retention at 30°C. were measured in a volumetric apparatus similar to that of Bailey and Johnson (2). Dough aliquots corresponding to 25 g. of flour were used. Gas production was measured in burettes inverted over saturated salt solution and gas retention over 23% potassium hydroxide solution. Readings were taken at 10-minute intervals.

Although it is realized that a strict comparison to baking conditions ends at the time the loaf goes to the oven (3 hours and 55 minutes under standard A.A.C.C. procedure), measurements were taken beyond this point on the assumption that the behavior of the dough at extended periods at 30°C. may parallel its behavior in the oven.

The formula used for baking is as follows: 3% yeast, 5% sugar, 1.75% salt, 0.1% ammonium dihydrogen phosphate, 0.3% malt, 4% nonfat milk solids, 3% shortening, and 0.001% potassium bromate.

The flour series examined represented six subseries of 16 flours each, from individual varieties of hard red Canadian spring wheats. The protein range for the entire flour series was from 7.9 to 18.6% on 13.5% moisture basis.

Results and Discussion

Effect of Sucrose. The effects on fermentation rates of additions of sucrose to a simple saltless dough with 3% yeast are shown in Fig. 1-A. The yeast used for all this work gave sharply defined double-maxima rate curves, as can be seen in the curve for the sugarless dough (0%).

Added sucrose raises the first maximum, lowers the second, and delays its appearance. With increasing amounts of sucrose the min-

imum becomes less pronounced until with 5% dosage it disappears altogether, thus giving a curve with only one maximum. From this maximum the rate falls off fairly sharply at first and then more gradually, indicating that fermentation of maltose may be partially responsible for the gassing rate at later time intervals. Of course the 5%-sugar curve may be entirely the result of sucrose fermentation, to the exclusion of maltose, but this appears unlikely from scrutiny of the intermediate curves. In general, the effect of sucrose in delaying the effective period of, and diminishing the rate of, maltose fermentation is quite clear.

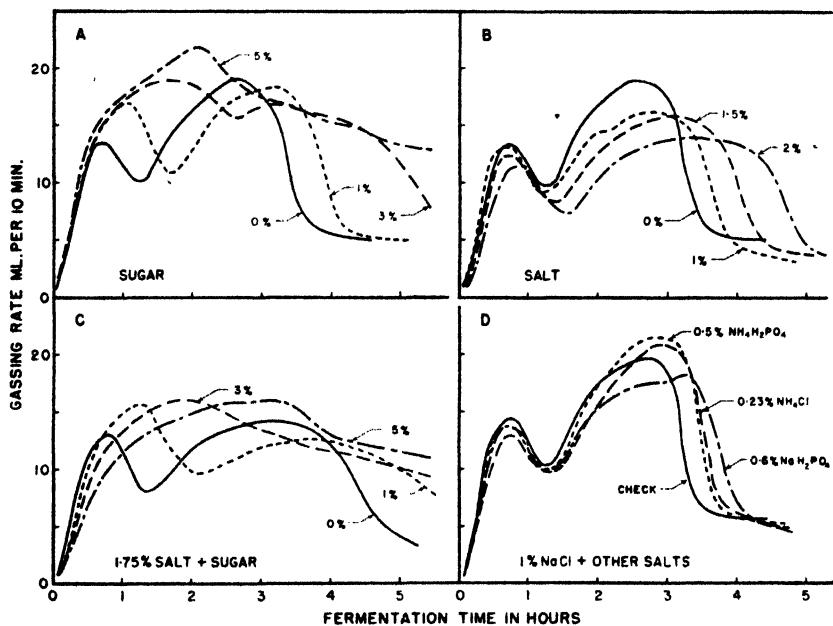


Fig. 1. Effects of various dough ingredients on gassing rates of doughs fermenting with 3% yeast. A. Sucrose dosages from 0 to 5%. B. Salt dosages from 0 to 2%. C. Sucrose dosages from 0 to 5% with 1.75% salt. D. Ammonium and phosphate salts with 1% sodium chloride.

The important thing to bear in mind with these rate curves is the total length of time over which a dough must gas in order to accomplish satisfactory rising during the pan-proof period. It is most essential to ensure good gassing between the third and fourth hours, the period during which the dough is proofing in the pan, because much of the conditioning effects of the dough during the mixing and fermentation period may be vitiated if in the last stage the gassing rate falls so low that the dough cannot become properly expanded. In Fig. 1-A both the sugarless and the 1%-sugar doughs exhibit a sharply declining rate of gassing during this critical period, and hence they

would not be adequately proofed by the end of the fourth hour and would therefore produce a small loaf of bread when proofed for a fixed time. On the other hand, both the 3%- and the 5%-sugar doughs maintain their gassing rates adequately during this period and hence could proof to the limit of their capacity to retain the gas.

While for purposes of conciseness and clarity, sugar dosages of 1, 3, and 5% only are shown in Fig. 1-A, intermediate values of 2 and 4% were also studied. These presented no anomaly and without graphical evidence it may be stated here that 2% sucrose is the minimum dosage capable of maintaining a relatively high gassing rate throughout the fourth hour.

Effect of Salt. The effects of varying salt dosages on gassing rates of 3%-yeast, sugarless doughs are shown in Fig. 1-B. Salt depresses the gassing rate but extends the period of relatively high gas production. Due to the relative size of the maxima, the effect is more noticeable on the latter part of the curve, which has been attributed to maltose fermentation, than on the initial part, which corresponds to sucrose fermentation. This has the effect of prolonging the time over which reasonably high gassing rates occur. For example, with 2% salt, without added sugar, the rate shows a broad maximum which extends from before the third hour to well beyond the fourth hour, thus providing stable continuous gassing during the pan-proof period. The rate, although less than with the lower salt dosages, averages about 13.5 ml. per 10 minutes, which, in a 100-g. formula, produces 300 ml. of carbon dioxide during the 55-minute period, sufficient to raise the dough properly if it has the capacity to retain the gas. By contrast the 1% curve shows that at the 3-hour time interval the gassing rate reaches its maximum and thereafter falls very sharply until at the fourth hour it is down to 5 ml. per 10 minutes, a rate wholly inadequate for proper proofing.

Maintenance of this adequate rate to the 4-hour point is accomplished by 1.75% (not plotted here) and 2.00% salt levels. These are the two levels most commonly used in commercial baking.

Combined Effects of Salt and Sugar. To determine the interaction of salt and sugar, both normally dough components under American baking practice, a study was made of the effects of sucrose at levels from 0 to 5%, on doughs having 1.50, 1.75, and 2.00% salt respectively. As the pattern of the gassing rate curves proved to be similar, only one is reproduced here, namely that at 1.75% salt with sugar at 0, 1, 3, and 5% levels. These curves are shown in Fig. 1-C.

It is interesting to note that while with 1% sugar the two maxima are clearly evident, at 3 and 5% sugar levels, the minimum has disappeared. Data not presented here show that this is true also for the

2% sugar level. As the two maxima are clearly evident with 3% sugar in a saltless dough (Fig. 1-A), and there is no indication of eradication of the minimum with any of the salt concentrations tested (Fig. 1-B), there must be a complementary effect due to the combined salt and sugar at this level of sugar.

These considerations together with other data too numerous to include here, Walden (15), indicate that the maximum gassing rate obtainable, with salt and sugar only, is an inverse function of the salt level. Increasing sugar merely delays attainment of this maximum set by the salt level and extends its duration.

Effects of Ammonium Salts. The stimulating effect of the ammonium ion on the rate of gassing is shown in Fig. 1-D. The curve marked "check" represents the rate with 3% yeast only. The data for the other three curves were obtained with doughs containing 1% sodium chloride, 3% yeast, no sugar, and amounts of ammonium chloride, ammonium dihydrogen phosphate, and sodium dihydrogen phosphate monohydrate respectively equivalent to 0.25% sodium chloride.

Despite the depressing effect of 1% sodium chloride (see Fig. 1-B), the small additional amount of the ammonium salts markedly increases the gassing rate at the second maximum. The two ammonium salts have almost identical effects in this respect. Sodium phosphate, on the other hand, depresses and extends the second maximum, behaving similarly to sodium chloride. It is thus evident that the effective ion is the ammonium ion, which confirms the conclusions of Larmour and Bergsteinsson (7).

Effect of Yeast Concentration. The combined effects of salt and sugar, in general, are to flatten out and extend the fermentation-rate curve, making it possible to achieve a well-sustained gassing rate through the pan-proof period, i.e., from the third to the fourth hour in the standard experimental baking procedure. The level of gassing will, however, depend on the amount of yeast present, and it is important, therefore, to choose a concentration that will ensure a sufficiently vigorous gassing rate to avoid the possibility of gassing becoming a limiting factor in the final loaf volume of the flour under test.

Two levels of yeast, i.e., 2 and 3%, were used with various sucrose dosages from 1 to 5%. Commercial and experimental practices correspond roughly to 2 and 5% sugar levels respectively, and these are the only values shown in Fig. 2. Other values being interjacent are omitted for purposes of simplification. The salt level of 1.75% was chosen as most nearly representative of baking practice and also one which extended the gassing rate to the 4-hour point.

The 3% yeast concentration starts the fermentation faster and the

rate goes higher in the initial phase than with the 2% concentration. After the first maximum has been passed at the end of 3 hours, three of these curves come close together and remain so until the 4.5 hour point when they diverge. These three curves are: 2% sugar, 3% yeast; 2% sugar, 2% yeast; and 5% sugar, 2% yeast. With the 2%-yeast level the yeast is the limiting factor in gas production during the 1.5 hour period from the 3-hour to the 4.5-hour points. With the 3%-yeast dough the high rate of fermentation achieved in the early phase exhausts the supply of added sucrose, and in the latter part of the period the limiting factor becomes the supply of maltose from the flour.

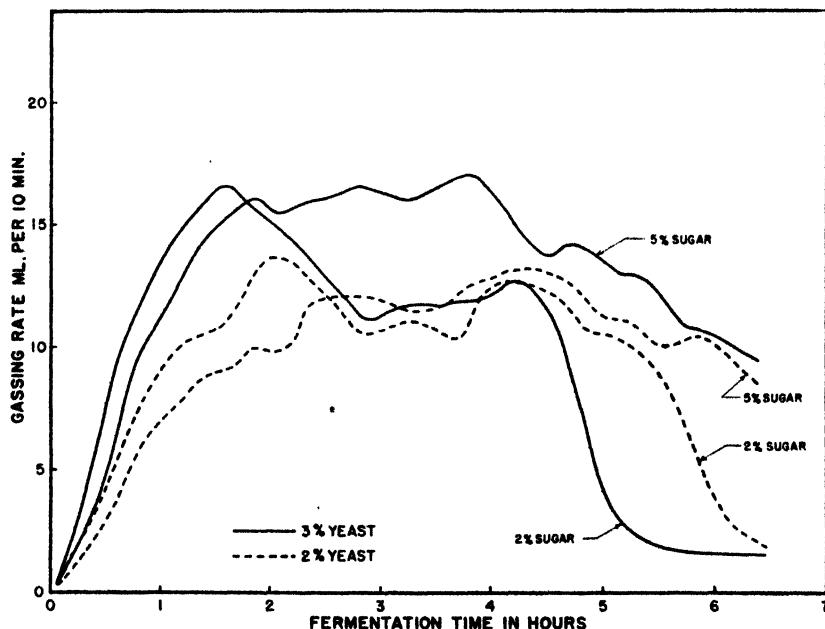


Fig. 2. Effects of sugar dosages on gassing rates of 1.75% salt doughs fermenting with 2 and 3% yeast.

With the 3%-yeast, 5%-sugar formula a high rate of gassing is maintained for 2 hours, i.e., between the second and fourth hours. With this formula it would be possible to ensure good gassing to the 5.5-hour point, because although by that time the rate has declined from the high of about 16 ml. per 10 minutes, it is falling slowly and is still at a higher value than the rates for the other three formulas during the critical period, i.e., between the third and fourth hours.

These data show that increased yeast tends to offset the depressing effects of increased salt; and that in order to ensure prolonged adequate gas production in doughs it is necessary to combine high salt, high

sugar, and high yeast concentrations. The levels of gassing shown here may not be necessary for commercial baking, but for experimental flour testing it is absolutely essential to provide conditions such that there are no factors likely to limit loaf volume other than the quality and strength of the flour itself. *The formula must ensure adequate gassing, especially throughout the pan-proof period, as otherwise the final volume may be merely a reflection of the failure of the formula rather than of the flour.*

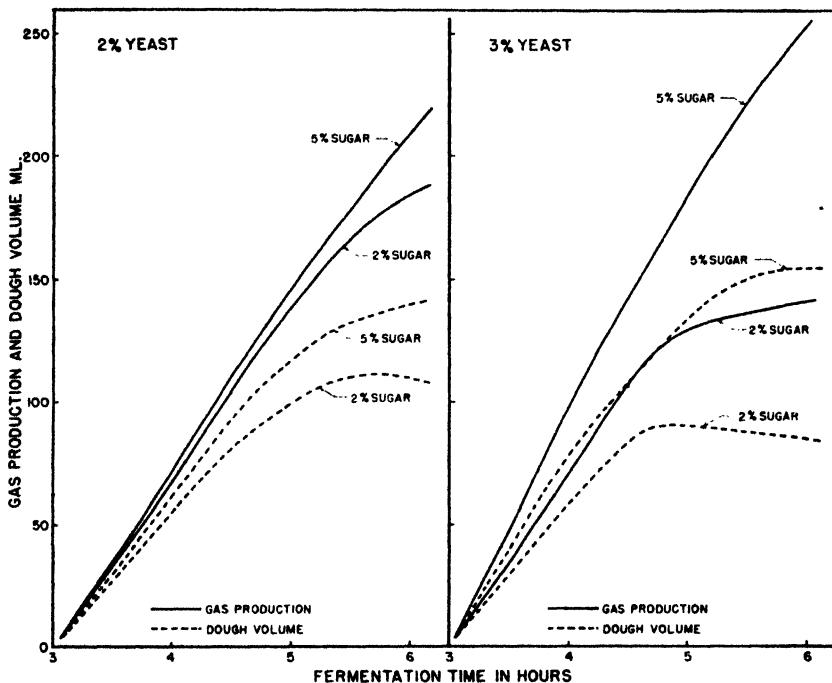


Fig. 3. Total gas production and dough volume changes of 1.75% salt doughs fermenting with 2 and 3% yeast at 2 and 5% sugar levels.

Dough Volume During Pan-proof Period. To test the foregoing conclusions further, the dough volumes and the total gas production of the doughs discussed in the preceding section were measured from the third hour to beyond the sixth hour. The results are shown graphically in Fig. 3.

Considering first the 2% yeast doughs, at the end of the normal proofing period, i.e., at the fourth hour, the total gas produced by the 5%-sugar formula is 72 ml. and by the 2% sugar formula, 68 ml., the two being practically equal. The dough volumes are 60 ml. and 56 ml. respectively. At this 4-hour point, therefore, the amount of sucrose originally added has no appreciable differentiating effect on either the

total gas production or the volume of the dough. Hence the limiting factor for dough volume must be the yeast concentration.

With the 3%-yeast formula the total gas production with 5% sucrose at the 4-hour point is 100 ml., while for the 2% sucrose it is 70 ml., which is virtually the same value as for both sugar concentrations with the 2%-yeast formulas. Here, however, there is a wide difference between the two sugar concentrations, the 5% formula producing far more carbon dioxide than any of the other three formulas. The differences are further emphasized by the dough volumes, because in the 3%-yeast formulas the 5% sugar gives far higher volume at this point, while the 2% sugar gives practically the same as the two formulas with 2% yeast.

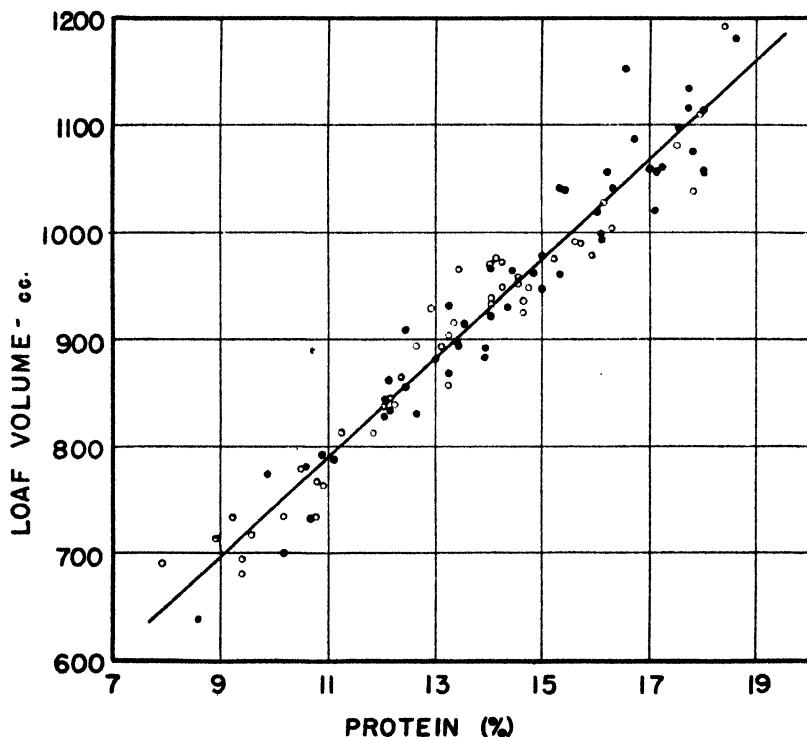


Fig. 4. Scatter diagram showing relation between loaf volume and protein content.

Further differences in the behavior of these four formulas are revealed by examination of the graphs beyond the 4-hour point. With 2% yeast the dough volumes at 5 hours are 118 and 100 ml. for the 5%- and the 2%-sugar formulas respectively, while with the 3% yeast they are 134 and 90 ml. for the 5%- and the 2%-sugar formulas re-

spectively. Here the 3%-yeast, 2%-sugar formula reflects the early exhaustion of the sugars, as shown in Fig. 2.

Thus, the high yeast, high salt, high sugar formula shown to be the most suitable on the basis of gassing power actually does increase the dough volume achieved during the pan-proof period as a result of its more adequate gassing rate.

Application to Baking. A series of 96 flours, baked by a high yeast, high salt, high sugar formula as described earlier showed exceedingly high correlation between loaf volume and protein content as evidenced by the data in Table I and Fig. 4.

TABLE I
STATISTICAL DATA FOR LOAF VOLUME AND PROTEIN CONTENT

	Entire series	Apex	Marquis	Rival	Renown	Regent	Thatcher
Mean protein of flour, %, \bar{x}	13.88	13.96	13.46	13.58	14.05	14.14	14.09
Mean loaf volume, cc., \bar{y}	923.4	905.2	908.5	901.3	930.1	935.3	959.8
Correlation coefficient, r_{xy}	+0.971	+0.991	+0.979	+0.982	+0.988	+0.994	+0.963
Regression coefficient, b_{yx}	+46.27	+37.08	+40.88	+52.64	+42.54	+46.04	+54.34
1% point	0.254	0.623	0.623	0.623	0.623	0.623	0.623

Although protein content is a strict measure of gluten quantity and not quality, this very close relationship indicates that there are few other factors concerned. It must be concluded, therefore, that the gluten quality of the flours was virtually the same throughout the series and that the gas production of the doughs was not a variable of any significance in relation to loaf volume.

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THE MOLD FLORA OF STORED WHEAT AND CORN AND ITS RELATION TO HEATING OF MOIST GRAIN¹

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ABSTRACT

All of more than 100 samples of corn, and several samples of wheat, collected from commercial lots were found to bear molds able to grow at relatively low moisture contents. The number of molds on corn increased with increasing moisture content of the seed. In both corn and wheat stored in vacuum bottles at different moisture contents, mold population and temperature increased with increasing moisture. At moisture contents favorable to their growth, the molds caused the temperature to rise to within a few degrees of the maximum the molds could endure. Two of the molds common on moist stored grain, *Aspergillus candidus* and *A. flavus*, grew on and heated moist sound wheat as rapidly, and to as high a temperature, as they did autoclaved wheat, while *A. glaucus* caused a higher rise in temperature of autoclaved than of sound wheat. Autoclaved moist wheat inoculated with 200,000 spores of *A. flavus* per gram heated to 45°C. in two days, while inoculated with only 0.2 spore per gram heated to a comparable temperature in nine days, indicating that if conditions for mold growth are favorable, the amount of inoculum originally present may have only a minor effect on eventual heating. Under the conditions of the tests, none of the several fungicides used eliminated molds from moist, stored wheat, and thiourea appeared to be less toxic to *A. candidus* than to other common molds.

That molds may be responsible for heating and various deteriorative changes in moist stored seeds and other organic products has been shown by a number of workers (2, 3, 4, 6, 7, 8, 10, 13). Much of the work in this field up to 1944 has been summarized by Semeniuk and Gilman (12). Koehler (5) and Semeniuk, Nagel, and Gilman (13) have determined with considerable precision the moisture contents of

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seeds and relative humidities of air required for germination and growth of a number of mold species common on stored corn. The present work aimed to explore some of the major factors known, or presumed, to influence the growth of molds on stored corn and wheat, and thus to furnish a partial background of information for more intensive and critical studies.

Materials and Methods

In 1945, 100 samples of commercial corn, each weighing about four oz., were collected by Federal Grain inspectors at each of five terminals: Cedar Rapids, Iowa; Omaha, Nebraska; Minneapolis, Minnesota; Chicago, Illinois; and Toledo, Ohio. These were sent to the University Farm, St. Paul, in previously sterilized paper envelopes or bags. In 1946 a smaller number from various midwestern states were submitted by the Research Division of The Quaker Oats Co. Most of these were placed in one-pound tins as soon as collected, sealed, and shipped by air mail. The majority of them were received and assayed for molds the day after they were collected. No systematic collection of wheat was made, the lots tested having come from various sources as indicated in the presentation of results.

The mold assays were made by grinding the grain in a Wiley mill equipped with a 40-mesh sieve. The mill was cleaned between lots, and the first portion ground was discarded, a practice which preliminary tests showed would prevent any serious contamination from the preceding one. The resulting meal, collected in sterile paper bags, was cultured according to the technic described by Christensen (1). Several culture media were tested, including malt-salt, Czapek's, acid potato dextrose, Smith-Humfeld, and corn-meal agars; the malt-salt medium was found to be preferable, in that it generally yielded a larger number of a greater variety of molds than any of the others.

Water content of the grain was determined by drying two or three 25-g. portions to constant weight at 105°C. In comparative tests this was found to give results averaging within 0.1% of those obtained by the more precise oven-vacuum method. To determine the ability of various molds to heat grain, the seed, after any preliminary treatment as described under the individual experiments, was conditioned to the required moisture content, and 200-g. portions were placed in pint vacuum bottles; or the grain was placed in vacuum bottles, water added to bring it to the required moisture content, the bottles closed with rubber stoppers, placed in a horizontal position, and rotated and shaken occasionally during a 24-hour period, by which time it was assumed that the water was evenly distributed through the grain. The rubber

stoppers were then replaced with sterilized cotton plugs to permit air exchange.

This method is essentially the same as that used by some of the earlier workers (2, 3). It is, of course, less accurate than the methods more recently described (9, 11) in which adiabatic conditions are approached. The most serious objection to it is that the original moisture content of the material may be reduced by loss of vapor through the cotton plugs, or increased by the metabolic water from mold growth. Also, with time, the water tends to become unevenly distributed within the container. Recent work (8) indicates that it may be extremely difficult to maintain a uniform moisture content in grain on which molds are vigorously growing, even when the grain is ventilated with air of constant relative humidity. For these reasons, in the present study only approximate relationships between moisture content and mold growth could be determined.

Results

Mold Population of Commercial Samples of Corn and Wheat. The mold population of 120 samples of corn of the 1945 crop, and of 15 samples from the 1946 crop, is summarized in Tables I and II.

TABLE I
MOLDS PER GRAM IN CORN SAMPLES FROM FIVE MIDWEST
TERMINALS, 1945 CROP

Grade	Maximum moisture content permissible, wet weight basis	Number of samples	Molds per gram	
			Range	Average
1	14.0	9	0-48,000	12,000
2	15.5	17	1450-522,000	96,000
3	17.5	21	0-1,470,000	262,000
4	20.0	16	5000-1,350,000	390,000
5	23.0	18	25,000-2,270,000	940,000
Sample		39	0-4,375,000	830,000

The species of molds were mainly those found by previous workers (4, 5, 13, 14) in corn and corn meal. In many samples typical "cob rotting" organisms were prevalent, such as *Fusarium moniliforme*, *Nigrospora sphaerica*, *Diplodia zeae*, *Cephalosporium acremonium*, and *Penicillium* spp. Koehler (5) showed that these are not able to grow in corn with a moisture content below 21 to 23%, and so ordinarily are of little significance in stored corn. In other samples *Aspergillus candidus*, *A. flavus*, *A. glaucus*, *A. niger*, *A. fumigatus*, and *A. versicolor* predominated. According to Thom and Raper (15) most of these are so-called "group" species, and each includes a number of different

TABLE II
MOLDS PER GRAM IN CORN FROM SEVEN MIDWEST TERMINALS, 1946 CROP

Source of sample	Moisture content	Molds per gram	Principal species of molds	Percentage of population
Akron, Ohio	16.0	8,000	<i>Monilia candida</i> <i>Aspergillus glaucus</i>	60 40
Akron, Ohio	17.3	110,000	<i>Fusarium moniliforme</i> <i>A. glaucus</i>	90 10
Cedar Rapids, Iowa	20.0	1,000,000	<i>M. candida</i>	95
Akron, Ohio	20.1	142,000	<i>F. moniliforme</i> <i>M. candida</i> <i>A. niger</i> and <i>A. flavus</i>	70 20 10
Akron, Ohio	20.2	1,600,000	<i>M. candida</i>	100
Lewisville, Ind.	20.5	20,000	Penicillium sp.	90
Chicago, Ill.	20.6	1,000,000	<i>M. candida</i>	100
Akron, Ohio	20.7	500,000	<i>M. candida</i>	99
Ames, Iowa	20.8	1,300	Penicillium sp. <i>A. niger</i> and <i>A. flavus</i>	50 50
Chicago, Ill.	21.5	1,800,000	<i>M. candida</i>	100
Sulfur Springs, Ind.	22.2	5,000	<i>M. candida</i> Penicillium sp. <i>A. niger</i> and <i>A. flavus</i>	30 40 30
Akron, Ohio	22.4	134,000	<i>M. candida</i>	95
Hallville, Ill.	22.9	96,000	Penicillium sp.	90
Cedar Rapids, Iowa	28.3	47,000	Mucor sp.	90
Cedar Rapids, Iowa	30.0	60,000	Mucor sp. <i>F. moniliforme</i>	85 15

types. In general, they are able to grow in materials whose water content is in equilibrium with an atmospheric relative humidity of 75 to 90%, and their occurrence in large numbers on many of these corn samples was only to be expected from the work cited above. Penicillium of undetermined species was found in nearly all of the samples, often being the dominant organism. Mucor spp. and Hormodendrum sp. predominated on a few of the lots, and were present on a majority of them.

The yeast-like organism, *Monilia candida* (apparently identical with *Candida albicans*), predominated in seven of the 15 1946 samples, growing on the outside of the seeds as an inconspicuous crust that on white corn was nearly invisible. Where present in amounts of several

hundred thousand per gram, it gave a decided sour and unpleasant yeasty odor to the corn. Pure cultures of the organism on various agar media have the same odor. Preliminary studies indicate that it will grow fairly rapidly on corn with moisture contents of 22 to 25%, but is not able to grow at moisture contents below 20%; also that it is a facultative anaerobe, able to grow slowly in moist corn stored in an atmosphere of carbon dioxide. It has since been found on various other lots of corn of the 1946 and 1947 crops.

The molds found on several samples of wheat are shown in Table III.

TABLE III
MOLDS PER GRAM IN SEVERAL SAMPLES OF WHEAT FROM VARIOUS SOURCES

Source	Crop year	Condition of wheat	Molds per gram	Kinds of molds
South Dakota	1945	Sound	2,040	Chiefly <i>Aspergillus glaucus</i>
South Dakota	1946	Sound	3,150	Chiefly <i>A. glaucus</i> and <i>Penicillium</i> sp.
Minnesota	1945	Sound	3,460	<i>A. glaucus</i> and <i>A. candidus</i>
Minnesota	1947	Immature	5,000	<i>Penicillium</i> sp., <i>A. glaucus</i> , <i>Mucor</i> sp., and <i>Alternaria</i> sp.
Southwestern U. S.	1946	Sound	2,600	<i>A. glaucus</i> , <i>A. candidus</i>
Southwestern U. S.	1946	Sick	66,000	<i>A. niger</i> , <i>A. flavus</i> , <i>A. candidus</i> , <i>Penicillium</i> sp., <i>Mucor</i> sp.

In general, a much less diverse flora was found on wheat than on corn, most probably because wheat normally is harvested with a much lower moisture content than corn. The principal organisms on all of the wheat samples tested were the species of *Aspergillus* adapted to low moisture contents, such as *A. glaucus*, *A. candidus*, and *A. ochraceus*, with *Penicillium* sp. and *A. flavus* being found occasionally in small numbers. The one sample of "sick" wheat tested had a much higher population of a more varied mold flora than the sound wheats.

Relation of Moisture Content and Mold Growth to the Heating of Corn. Corn of the 1945 crop which had been stored in a dry basement for nearly a year was moistened to six different water contents and 200 g. were placed in pint vacuum bottles, two bottles being used for each water content. The corn was not inoculated with molds, but bore a rather heavy and varied natural mold flora. The rise in temperature of this corn and the molds associated with it are shown in Table IV.

To determine the effect of some of the individual molds upon the heating of moist corn, spores from pure cultures were inoculated onto autoclaved corn which had been conditioned to various moisture con-

TABLE IV
HEATING OF NONINOCULATED CORN AT VARIOUS MOISTURE CONTENTS—
NATURAL MOLD FLORA PRESENT

Water, per cent, wet weight basis	Temperature, °C.			Molds present at end of test
	4 Days	8 Days	12 Days	
16	24	25	24	<i>Aspergillus glaucus</i> , sparse conidia
18	25	26	26	<i>A. glaucus</i> , perithecia abundant
20	26	27	29	Mainly <i>A. glaucus</i> , some <i>A. candidus</i>
22	35	47	50	<i>Mucor</i> , <i>A. flavus</i> , <i>A. candida</i>
24	33	45	50	<i>A. terreus</i> , <i>A. flavus</i>
26	45	51	53	<i>A. fumigatus</i>

tents with hot sterile water after the autoclaved corn had been placed in sterile vacuum bottles. The temperature of this corn dropped to that of the room in approximately 24 hours. The results are presented in Tables V and VI.

TABLE V
INFLUENCE OF THREE DIFFERENT MOLDS ISOLATED FROM HEATING CORN
UPON THE HEATING OF AUTOCLAVED CORN AT THREE
DIFFERENT MOISTURE CONTENTS

Mold	Days after inoculation	Moisture content, per cent of wet weight		
		18	19	20-21
<i>Aspergillus</i> <i>candidus</i>	4	40 ¹	42	45
	20	44	44	45
<i>A. flavus</i>	4	46	43	—
	20	45	43	—
Penicillium sp.	4	29	29	28
	20	34	34	35

¹ Temperature, °C.—room temperature 22°-25°C.

TABLE VI
HEATING OF AUTOCLAVED CORN AT DIFFERENT MOISTURE CONTENTS,
INOCULATED WITH DIFFERENT MOLDS ISOLATED
FROM HEATING CORN

Mold	Water, per cent of wet weight				
	14	16	18	20	22
<i>A. candidus</i>	20 ¹	21	26	43	40
<i>A. flavus</i>	22	22	22	41	—
<i>A. terreus</i>	20	20	21	34	44
Control (not inoc.)	21	—	21	—	21

¹ Temperature after 12 days, °C.

Supplementary tests, in which these molds were grown in pure culture on nutrient agar in petri dishes exposed continuously to various temperatures, indicate that the maximum temperature induced by each mold on autoclaved corn in vacuum bottles was within 2°-3°C. of the maximum temperature at which the organism concerned could grow.

Relation of Moisture Content and Mold Growth to the Heating of Wheat. Two varieties of wheat—Marquis, grown in Montana and nine years old at the time of the tests, germinating 90% (a number of seeds germinated slowly, indicating a decreasing viability), and Rival, from Minnesota, one year old and germinating 95%, with normal vigor—were conditioned to moisture contents of 20.0, 22.5, and 25.0%, 200 g. of each variety at each moisture content, placed in each of two vacuum bottles, and stored in the laboratory. After 11 days' storage, the temperatures in the bottles were recorded, the seed in each bottle was poured into a sterile paper bag held closely around the neck of the

TABLE VII

RELATION OF WATER CONTENT TO MOLD INCREASE AND TEMPERATURE
RISE IN TWO VARIETIES OF WHEAT STORED IN VACUUM BOTTLES
11 DAYS—ROOM TEMPERATURE 22°-23°C.

Wheat	Water, per cent of wet weight	Temperature, °C.	Molds per gram	Species	Per cent
Marquis	20.0	25.0	720,000	<i>A. glaucus</i>	90
				Penicillium	5
				<i>A. candidus</i>	5
Rival	20.0	23.5	880,000	<i>A. glaucus</i>	80
				Penicillium	10
				<i>A. candidus</i>	5
				Others	5
Marquis	22.5	25.0	2,235,000	<i>A. glaucus</i>	50
				Penicillium	40
				<i>A. candidus</i>	10
				and <i>A. niger</i>	
Rival	22.5	25.0	1,623,000	Penicillium	40
				<i>A. candidus</i>	30
				<i>A. flavus</i>	20
				<i>A. glaucus</i>	10
Marquis	25.0	39.0	173,000,000	<i>A. flavus</i>	80
				<i>A. candidus</i>	10
				Penicillium	5
				<i>A. glaucus</i>	5
				and <i>A. niger</i>	
Rival	25.0	29.0	5,150,000	<i>A. flavus</i>	80
				Penicillium	15
				<i>A. niger</i>	5

bottle to prevent undue escape of spores, and mixed by shaking the bag. Several grams of this seed were removed from the bag, dried at room temperature in a sterile dish, then ground and the mold population determined. The results are given in Table VII.

An approximately similar mold flora developed on the two varieties, and the number of molds per gram were roughly proportional to the rise in temperature. Marquis heated more rapidly than Rival, and a larger population of molds developed on it, presumably because of its greater age, lower vitality, and thus greater susceptibility to invasion by molds.

To compare the rate of temperature increase induced by certain of the molds on living wheat with that on autoclaved wheat, a Montana hybrid wheat almost free of internal mold infection, germinating 95%, was surface disinfected by using a 0.5% solution of sodium hypochlorite as conditioning water. Previous tests on this seed lot had indicated that this would free it almost completely of molds, although this has not been true of other seed lots so tested. Rival wheat, also germinating 95%, was autoclaved at 15 lbs. pressure for 30 minutes, placed in sterile vacuum bottles, and conditioned with hot sterile water. After the water was presumed to be evenly distributed, by which time the temperature of the seed had dropped to that of the room, the two different grains were inoculated with three different molds. The results are shown in Tables VIII and IX.

TABLE VIII
HEATING OF AUTOCLAVED RIVAL WHEAT WITH DIFFERENT MOLDS—
MOISTURE CONTENT 30% OF WET WEIGHT

	Temperature, °C., in duplicate bottles		
	5 Days	7 Days	8 Days
<i>Aspergillus glaucus</i>	26, 27	31, 34	38, 38
<i>A. candidus</i>	26, 32	43, 45	47, 47
<i>A. flavus</i>	43, 40	47, 47	45, 44

TABLE IX
HEATING OF SURFACE-DISINFECTED MONTANA WHEAT INOCULATED WITH
DIFFERENT MOLDS—MOISTURE CONTENT 30% OF WET WEIGHT

	Temperature, °C., in duplicate bottles			
	5 Days	7 Days	8 Days	14 Days
<i>Aspergillus glaucus</i>	27, 28	26, 27	25, 27	28, 31
<i>A. candidus</i>	44, 45	42, 43	44, 44	45, 46
<i>A. flavus</i>	45, 45	44, 45	44, 44	45, 46

Aspergillus glaucus heated the autoclaved grain faster, and to a higher final temperature, than it did the nonautoclaved grain. *A. candidus* and *A. flavus*, however, grew on and heated the nonautoclaved, living seed just as rapidly as they did the autoclaved seed. (This is in agreement with the results of Milner, Christensen, and Geddes (6) in which it was shown that *A. candidus* and *A. flavus* rapidly reduced the viability of wheat at 18% moisture, being fairly vigorous facultative parasites of such dormant seed, while *A. glaucus* reduced the viability much more slowly. This test suggests that seed "condition" may determine to some extent the growth of certain molds and thus may be an important factor in the storage of grain.)

Relation of Amount of Inoculum to Rate of Mold Increase and Temperature Rise of Moist Wheat. Rival wheat was autoclaved at 15 lbs. pressure for 30 minutes, and 200-g. lots were placed in sterile pint vacuum bottles. Inoculum was prepared by suspending spores of *Aspergillus flavus*, isolated from heating wheat, in sterile water to which one part of Vatsol (a wetting agent) to 10,000 parts of water had been added before sterilizing, to reduce the surface tension and facilitate a uniform suspension of spores. The original suspension was diluted 1:100, 1:10,000, and 1:1,000,000. The 1:10,000 dilution was cultured, and yielded 100 colonies per milliliter. Microscopic examination of the culture dishes after two to three days showed that over 90% of the colonies developed from single spores. A sufficient

TABLE X

INFLUENCE OF DIFFERENT AMOUNTS OF INOCULUM OF *Aspergillus flavus*
ON THE HEATING OF AUTOCLAVED CORN CONTAINING
25% MOISTURE, WET WEIGHT BASIS

Number of spores of <i>A. flavus</i> per gram of corn	Temperature, °C., average of two bottles									
	Days									
	2	3	4	5	6	7	8	9	10	
200,000	45	46	47	47	—	—	—	—	—	
2,000	30.5	39	46	46	—	—	—	—	—	
20	26	28	41	46	—	—	—	—	—	
0.2	25	25	26	26	28	33	40.5	46	—	
0.0	24	24	24	23	23	23	26.5	30	38.5 ¹	
(Control)										

¹ Heavily contaminated with *A. flavus*.

amount of the spore suspension of each dilution was added to the grain in each of two vacuum bottles to attain the desired moisture content. The bottles were then plugged with sterile rubber stoppers and occasionally rotated and shaken for 24 hours, after which the rubber stoppers

TABLE XI
EFFECT OF VARIOUS MOLD INHIBITORS UPON MOLD DEVELOPMENT IN, AND HEATING OF, MOIST WHEAT

Moisture content of grain, per cent of wet weight	Inhibitor	Concentration	When added	Effect on temperature	Condition of seed at end of test
25	Chloramine B	1:100	After temperature of grain had risen to 31°C.	Reduced temperature of grain to that of room (27°C.) for 5 days; temperature then rose again and in 10 days reached 42°C.	Heavily molded, <i>Mucor</i> sp. especially prevalent
30	Chloramine B	1:100	When moist grain was placed in vacuum bottle	Temperature rose slowly to 31°C.	Seed at bottom of flask bound together with mold
25	Spergon dust	1:1000	After conditioned grain was inoculated with <i>Aspergillus flavus</i>	Temperature rose to 45°C. in 14 days, only slightly slower rise than in controls	Heavily molded
25	P-toluenesulfonilamide	1:1000	After conditioned grain was inoculated with <i>Aspergillus flavus</i>	Temperature rose to 45°C. in 14 days, only slightly slower rise than in controls	Heavily molded
25	Chloramine B	1:1000	After conditioned grain was inoculated with <i>Aspergillus flavus</i>	Temperature rose to 45°C. in 14 days, only slightly slower rise than in controls	Heavily molded
25	Thiourea	1:500	When conditioned grain was placed in bottles; grain not inoculated with molds	Temperature rose to 36°C. in 18 days; controls rose to 46°C. in 10 days	Heavily molded with <i>Aspergillus candidus</i>

were replaced with sterile cotton plugs. Sterile water was added to the two controls. The temperature rise as related to spore load is given in Table X.

The controls remained at room temperature for eight days, at which time one began to heat, followed shortly after by the other. Both proved to be contaminated with *Aspergillus flavus*. In most of these experiments with moist grain in vacuum bottles it has proved difficult to keep molds from entering the control bottles, especially if thermometers are inserted through the cotton plugs at intervals to ascertain the temperature. In all bottles in this test, by the time the temperature had increased to the maximum, the seed was almost obscured by the spores of *A. flavus*.

Effect of Various Inhibitors on the Development of Molds and the Heating of Moist Wheat. To separate the deterioration of stored seeds induced by molds from that due to chemical processes in the seeds, themselves, it is necessary to obtain seed free of molds. The present test was not intended to measure the relative fungicidal efficiency of the compounds used, but rather to determine whether any of them might eliminate molds from grain stored under conditions near the optimum for mold growth. In some cases the fungicide was dusted on the wheat after it had been conditioned; in other cases it was added to moist seed which had already begun to heat. The results of these tests are presented in Table XI.

It is obvious that none of these compounds eliminated molds from the seed. Thiourea, considered by Milner, Christensen, and Geddes (8) to be the most effective moldicide of the compounds they tested, in the present test permitted a vigorous growth and sporulation of *Aspergillus candidus*, but apparently inhibited other molds almost completely. That a given compound may be effective against certain organisms but not others, or effective under certain conditions but not others, is too well known to need emphasis, and perhaps the chief value of the above tests is to indicate that this elementary concept must be kept in mind in work with stored seeds.

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FACTORS AFFECTING THE DETERMINATION OF THIAMINE AND RIBOFLAVIN IN ENRICHMENT PREMIXES CONTAINING FERRUM REDUCTUM¹

S. H. RUBIN, E. DE RITTER, E. FEBBRARO,
and F. W. JAHNS

ABSTRACT

Acid extraction of flour premixes containing ferrum reductum causes much greater losses of thiamine and riboflavin than are found when acid solutions of the vitamins are shaken with ferrum reductum under identical conditions. This difference arises from the fact that the vitamin losses occur chiefly during the interval in which the vitamins are going into solution. The effects of pH, specific buffer, and time of extraction on such vitamin losses from flour premixes are similar to those previously reported in model experiments with vitamin solutions wherein higher ratios of iron to vitamin were employed.

Increasing the acid concentration in the extraction of a flour premix containing ferrum reductum decreases the thiamine and riboflavin losses. If such premixes are extracted at 100°C. with a 0.1 *N* acid solution of cystine, at least 1.5 mols of cystine must be used per mol of iron to avoid vitamin losses.

Corn grits premixes containing ferrum reductum and vitamins in a limestone base also suffer high losses of thiamine and riboflavin during acid extraction. The high ratio of limestone to vitamins has little effect on the vitamin losses, in contrast to the marked reduction in these losses when similar quantities of flour or starch are mixed with a flour premix sample prior to hot acid extraction.

Thiamine and riboflavin are partially destroyed when flour enrichment premixes or other products containing large amounts of ferrum reductum are assayed by extraction in the usual acid extractants (3). No such losses occur when the iron is present as ferrous or ferric salts. The means devised to circumvent this effect in the presence of ferrum reductum include extraction at pH 6-6.5, where little or no iron goes into solution, and extraction with acid containing cystine, which is apparently reduced preferentially in place of thiamine or riboflavin. Other evidence has been adduced which indicates that the effect on these vitamins is reductive.

It has been noted in the course of this work that the *magnitude* of the vitamin losses from flour premixes is considerably greater than from model solutions of comparable concentration containing only the vitamins and iron. The present report details experiments bearing on this point as well as data on the effect of ferrum reductum in corn meal premixes and in enriched flour.

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Materials and Methods

Materials. The flour enrichment premixes were "double-strength" commercial premixes from three different manufacturers, containing, according to label claim, the following amounts of vitamins and iron, expressed in mg. per oz., in a starch base: thiamine, 760; riboflavin, 460; niacin, 5480; iron, 4800.

The corn meal premixes were laboratory batches containing limestone as a source of calcium and as a carrier for the vitamins and iron. Other laboratory-prepared premixes, as well as solutions, were made with U.S.P. crystalline vitamins and U.S.P. ferrum reductum, unless otherwise specified.

Analytical Methods. Thiamine was determined in premixes in direct dilutions, i.e., without the Decalso step, by the thiochrome method of Hennessey (4), which gave results equal to or slightly higher than colorimetric assays by the procedure of Hochberg, Melnick, and Oser (5). The latter is affected in some cases by inhibitors which can be removed by Decalso treatment (3).

Riboflavin was determined by direct fluorometric measurement with a hydrosulfite blank (3), and iron was determined colorimetrically by the A.A.C.C. α, α' -dipyridyl method (1).

Experimental Results

Comparison of Solid Vitamins and Solutions. In studies of the mechanism of the destruction of thiamine and riboflavin by acid extraction in the presence of ferrum reductum, it was found that shaking an acid *solution* of thiamine and riboflavin with ferrum reductum—with all components at the same concentrations as in the acid extraction of a flour premix—caused much smaller vitamin losses than were found in the case of flour premixes (cf. Table I). Since it appeared possible that the ingredients of the premixes might be enhancing the destructive effect of the ferrum reductum, the above experiment was repeated with C.P. ferrum reductum and also with starch and starch plus niacin added with ferrum reductum to the vitamin solution in premix proportions before shaking. Rice, wheat, and corn starches were also compared, but in no case was the vitamin recovery decreased by addition of one or a combination of these ingredients. Hence, the quantitative difference in the effect of ferrum reductum plus acid on premixes and vitamin solutions appears to lie in the fact that the vitamins are present in the one case as solids and in the other case in solution. To test this point further, dry mixtures were prepared with the same ingredients as in the above experiments, ranging from complete premixes to mixtures of a single vitamin and ferrum reduc-

tum. After shaking under the same conditions, thiamine and riboflavin losses were in all cases of the same order of magnitude as for the commercial premix and much greater than for comparable vitamin solutions.

TABLE I

DESTRUCTION OF THIAMINE AND RIBOFLAVIN BY FERRUM REDUCTUM DURING 30-MINUTE EXTRACTION IN 0.1 N SULFURIC ACID SOLUTION AT 24°C.—COMPARISON OF CRYSTALLINE VITAMINS AND VITAMIN SOLUTIONS

Method of adding vitamins	Content of test sample					Vitamin recovery	
	Thiamine mg.	Riboflavin mg.	Niacin mg.	Ferrum reductum mg.	Starch mg.	Thiamine %	Riboflavin %
0.150 g. commercial flour premix	4.0	2.6	28.6	27.8	87 (Wheat)	47	35
Vitamin solution	4.0	2.6	0	28 (USP)	0	92	93
Vitamin solution	4.0	2.6	0	28 (CP)	0	91	96
Vitamin solution	4.0	2.6	0	28	85 (Corn)	94	96
Vitamin solution	4.0	2.6	29	28	85 (Corn)	91	97
Vitamin solution	4.0	2.6	29	28	85 (Rice)	94	96
Vitamin solution	4.0	2.6	29	28	85 (Wheat)	93	99
Crystalline vitamins ¹	4.0	2.6	29	28	85 (Wheat)	45	42
Crystalline vitamins	4.0	2.6	29	28	0	41	42
Crystalline vitamins	4.0	2.6	0	28	0	46	43
Crystalline vitamins	4.0	0	0	28	0	40	—
Crystalline vitamins	0	2.6	0	28	0	—	39

¹ Mixtures were prepared by shaking the dry ingredients in the flask before extraction.

Effect of pH. In view of the unusual effects of pH and buffer ions on the action of ferrum reductum on solutions of thiamine and riboflavin reported by De Ritter and Rubin (3), and the differences between solid vitamins and solutions given in Table I, a study was made of the effect of pH in these buffers on vitamin losses from flour premix. Acetate buffer was studied over the range 3.0 to 6.5 and McIlvaine's phosphate-citric acid buffer series from pH 2.2 to 8.0 (2). In each series, 0.150 g. of premix was shaken 30 minutes at 24°C. with 300 ml. of buffer solution. The acetate buffer series was prepared by titrating 20, 30, 40, 50, and 60 ml. of 2.5 M sodium acetate with 5 N sulfuric acid solution to pH 3, 4, 5, 6, and 6.5, respectively, before dilution to 300 ml. For the phosphate-citrate buffer, 100 ml. of mixed buffer solutions at the desired pH were diluted to 300 ml. After shaking the flour premix with these solutions, diluting to 500 ml. and filtering, the pH of each extract was measured. The pH in each case was within 0.1 of the original value except for the lowest pH in each series which increased from 3.0 to 3.4 in the acetate buffer and from 2.2 to 2.4 in the phosphate-citrate buffer. Thiamine, riboflavin, and iron were

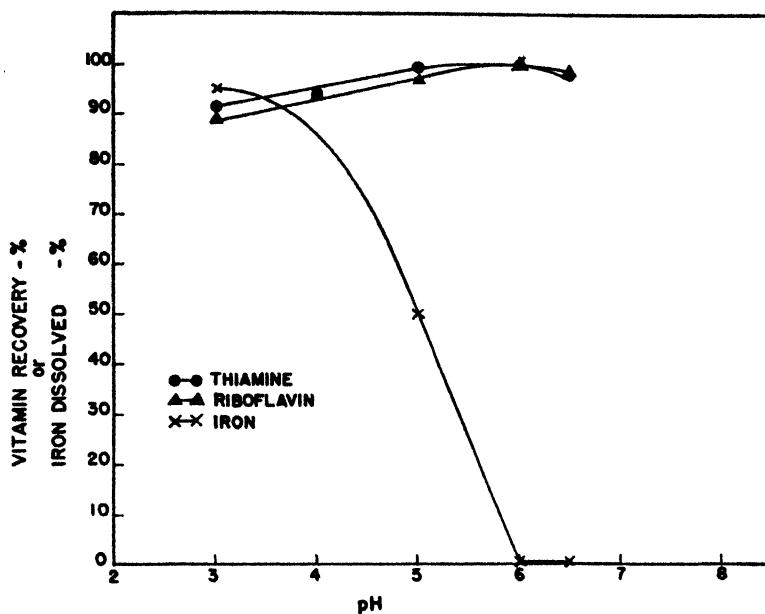


Fig. 1. Effect of pH on (a) recovery of thiamine and riboflavin and (b) solubility of iron in extraction of flour premixes containing ferrum reductum with acetate buffer.

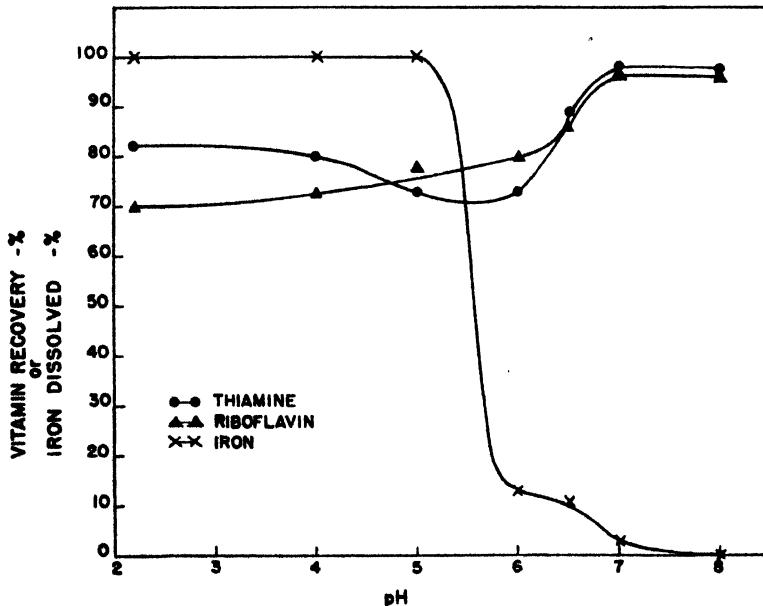


Fig. 2. Effect of pH on (a) recovery of thiamine and riboflavin and (b) solubility of iron in extraction of flour premixes containing ferrum reductum with phosphate-citrate buffer.

determined in each extract. The vitamin recovery and the extent of solution of the ferrum reductum are shown in Fig. 1 for the acetate and in Fig. 2 for the phosphate-citrate buffers. At lower pH values, vitamin recoveries are much higher in the acetate than in the phosphate-citrate buffer. In the former, recoveries are practically complete above pH 5, whereas in the latter, complete recoveries are obtained only at a pH of about 7. The ferrum reductum in the premix is more soluble in the phosphate buffer, but there is no direct correlation between the amount of iron dissolved and the amount of vitamins destroyed. In both buffers, there is a sharp increase over a narrow pH range in the amount of iron dissolved, but no appreciable change in the vitamin recovery.

Effect of Time of Shaking in 0.1 N Sulfuric Acid Solution. The rate of loss of thiamine and riboflavin in extraction of flour premixes with 0.1 N sulfuric acid solution at 24°C. was determined by shaking

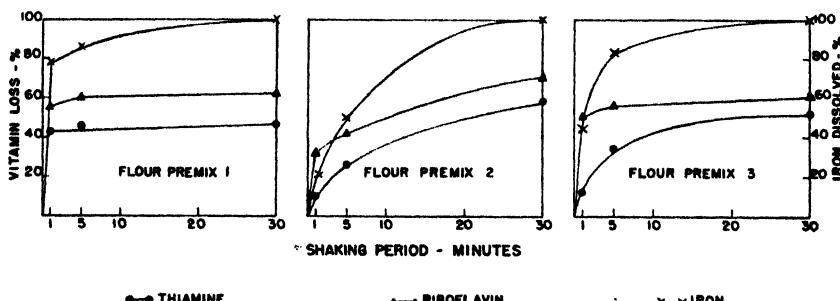


Fig. 3. Effect of time of shaking in 0.1 N sulfuric acid solution at 24°C. on (a) loss of thiamine and riboflavin and (b) extent of solution of iron from flour premixes containing ferrum reductum.

0.150 g. samples of three commercial premixes with 300 ml. of 0.1 N sulfuric acid solution for 1, 5, and 30 minutes. Vitamin losses and amounts of iron dissolved are shown in Fig. 3. It is evident that the destruction of both vitamins and the solution of the ferrum reductum occur rapidly. In this case, there appears to be a definite correlation between vitamin losses and iron solubility since all three curves for each premix follow the same general pattern.

Effect of Acid Concentration. Since it appears that the vitamin losses occur chiefly during the interval in which the vitamins are going into solution, it was of interest to determine whether increasing the concentration of the acid extractant would increase the rates of solution and consequently decrease the thiamine and riboflavin losses. Thus 0.150 mg. of a flour premix containing ferrum reductum were shaken 30 minutes at 24°C. with 100 ml. of acid. The effect of the acid concentration on the vitamin losses is shown in Table II. Both thiamine

and riboflavin show progressively higher recoveries with increasing acid concentration, the rate of increase in recovery being somewhat greater in the case of thiamine.

TABLE II

EFFECT OF ACID CONCENTRATION ON VITAMIN LOSSES FROM FLOUR PREMIX
(Extraction with sulfuric acid solution at 24°C.)

Acid normality	Vitamin recovery	
	Thiamine	Riboflavin
0.1	44	40
0.2	51	44
0.3	56	45
0.5	64	53
1.0	73	60
2.0	83	68

Minimum Amount of Cystine Required. Since a large excess of cystine in acid solution has been shown (3) to protect thiamine and riboflavin in flour premixes from destruction by acid plus ferrum reductum, experiments were performed to determine the minimum cystine/iron ratio required for complete protection. Thus 0.150 g. of flour premix was extracted 30 minutes at 100°C. with 300 ml. of 0.1 N sulfuric acid solution containing graded amounts of cystine as given in Table III. Thiamine and riboflavin assays of filtered extracts indicate that 1.5 mols of cystine per mol of iron is sufficient in all cases.

TABLE III

MINIMUM AMOUNT OF CYSTINE REQUIRED FOR HOT ACID EXTRACTION
OF FLOUR PREMIXES CONTAINING FERRUM REDUCTUM

Mol cystine per mol iron	Vitamin recovery			
	Thiamine		Riboflavin	
	Premix 1	Premix 2	Premix 1	Premix 2
0	%	%	%	%
0.15	88	71	76	68
0.30	99	87	91	98
0.75	100	96	92	97
1.5	99	95	96	100
3.0	100	98	100	99
	99	98	98	100

Stoichiometrically 1 mol of cystine is required in the oxidation of 1 mol of Fe° to Fe^{++} . However, an excess of cystine up to 3 mols per mol of iron does not interfere.

Extraction of Corn Meal Premixes. The effect of acid extraction on another product containing a relatively large amount of iron is

illustrated by the data in Table IV on corn grits premixes. Because of the high concentration of limestone in these premixes, it was necessary to use 500 ml. of 0.2 *N* sulfuric acid solution per gram of premix to keep the pH down to a level (approx. 1.0) at which no thermal destruction of vitamins would occur at 100°C. Extraction with an acid solution of cystine at 100°C. and with pH 6.0 acetate buffer, both of which are safe in the presence of ferrum reductum, was used as a basis of comparison. The premixes containing ferrum reductum suffered thiamine losses of 19 and 30% and riboflavin losses of 35 and 38%, respectively, during hot acid extraction. No vitamin loss was caused by acid extraction of the premixes containing sodium iron pyrophosphate.

TABLE IV
COMPARISON OF EXTRACTION PROCEDURES FOR CORN MEAL PREMIXES

Corn meal premix no.	Type of iron	Extractant		
		0.2 N H ₂ SO ₄ at 100°C. ¹	0.2 N H ₂ SO ₄ + cystine at 100°C. ¹	pH 6 acetate buffer at 24°C.
THIAMINE CONTENT—mg./oz.				
1	Ferrum reductum	3.9	4.7	4.9
2	Ferrum reductum	7.9	11.5	11.1
3	Sodium iron pyrophosphate	5.4	5.2	5.2
4	Sodium iron pyrophosphate	11.3	11.7	11.3
RIBOFLAVIN CONTENT—mg./oz.				
1	Ferrum reductum	11.7	18.0	18.1
2	Ferrum reductum	10.0	16.0	16.0
3	Sodium iron pyrophosphate	18.1	18.2	18.1
4	Sodium iron pyrophosphate	16.0	15.9	16.0

¹ 3 mols of cystine in acid solution per mol of ferrum reductum.

Protective Effect of Flour. Acid extraction of enriched 80% extraction flour containing ferrum reductum has been shown by De Ritter and Rubin (3) to entail no loss of either thiamine or riboflavin. To determine whether this lack of effect is due to the protective effect of the flour itself, 0.150 g. samples of three flour premixes containing ferrum reductum were mixed with 10 g. of such a flour before extraction with 300 ml. of 0.1 *N* sulfuric acid solution at 100°C. Comparative results for these premixes with and without added flour are given in Table V. The addition of flour provides almost complete protection

to both thiamine and riboflavin which, in the absence of flour, are destroyed to the extent of 15-25% and 32-40%, respectively, under the same conditions.

TABLE V
PROTECTIVE EFFECT OF FLOUR DURING EXTRACTION OF FLOUR PREMIXES
(In 0.1 *N* sulfuric acid solution at 100°C.)

Flour premix no.	Vitamin recovery			
	Premix only		Premix + flour ¹	
	B ₁	B ₂	B ₁	B ₂
1	%	%	%	%
2	85	68	97	96
3	77	66	97	98
	75	60	98	98

¹ 10 g. of 80% extraction flour mixed with 0.150 g. of flour premix before extraction.

In a similar experiment in which 10 g. of wheat starch were used in place of the 10 g. of flour, the starch was found to be almost as effective as the flour in preventing the vitamin losses. Hence, it appears that this protective effect of flour is due to the fine, starchy particles and is probably a physical rather than a chemical action.

Discussion

Acid extraction of flour enrichment premixes containing ferrum reductum causes much greater destruction of thiamine and riboflavin than is found on shaking acid solutions of the vitamins with ferrum reductum at comparable concentrations. This difference cannot be attributed to any of the other premix ingredients, but arises solely from the fact that the vitamins are present in one case as solids and in the other case in solution. Hence, it appears that the vitamin losses occur chiefly during the period in which the vitamins are going into solution. The high initial rates of loss shown in Fig. 3 confirm this observation.

The effect of pH in acetate buffer over the range 3 to 6.5, and in phosphate-citrate buffer from 2.2 to 8, is similar for the flour premix to that observed by De Ritter and Rubin (3) for vitamin solutions shaken with a much greater excess of ferrum reductum. Vitamin losses from the premix are small in acetate buffer and practically negligible above pH 5.0. In phosphate-citrate buffer, vitamin recoveries are poor up to pH 6.5 and only approach 100% from pH 7 to 8. The extent of these losses at various pH levels cannot be correlated with the amount of ferrum reductum dissolved. In 0.1 *N* acid extraction of flour premixes at 24°C., however, the rate of loss of both vitamins appears to be directly related to the rate of solution of the ferrum

reductum. The progressive decrease in vitamin losses, which is observed when the concentration of the acid extractant is increased, is probably a result of changes in the relative rates of solution of the vitamins and ferrum reductum.

The losses of thiamine and riboflavin during hot acid extraction from corn meal premixes containing ferrum reductum are of the same order of magnitude as from flour premixes. The relatively high ratio of limestone substrate to vitamins appears to have little effect on the vitamin losses. That the substrate may have a definite effect on the vitamin losses is shown by the experiments in which flour or starch was mixed with flour premix before hot acid extraction, with the result that vitamin losses from the premix were reduced almost to zero. Since flour is able to protect thiamine and riboflavin in the premix, it is not surprising that the relatively small amount of ferrum reductum in an analytical sample of enriched flour does not cause vitamin destruction during acid extraction.

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COMPOSITION OF THREE VARIETIES OF KANSAS-GROWN WHEAT. MINERAL ANALYSIS OF WHEAT AND SOIL¹

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ABSTRACT

Three different varieties of wheat, grown in 13 different locations in Kansas over a three-year period, have been analyzed for protein, ash, potassium, phosphorus, calcium, magnesium, sodium, manganese, iron, and copper. Soil samples obtained from the same locations were also analyzed for the same constituents, except for sodium. Soil pH was also measured. Rainfall data, yield, and test weight were also reported.

The ash and mineral content of wheat grown in various locations within the state varies appreciably and is correlated in general with the available nutrients in the soil. Areas producing wheat of high mineral content produced the higher protein.

The mineral content was not greatly influenced by variety. The location at which the wheat was grown was much more important than variety. Localities producing high mineral content did so consistently, indicating that differences due to rainfall and other factors during the three-year period did not significantly affect mineral content.

The increased ash content of western Kansas wheats was not due to any one element, but was the combined result of increases in each of the major constituents of the ash. A very high manganese content of wheat grain was noted from certain areas in 1943 but not the following two years. Apparently conditions were favorable for a maximum intake of manganese in these areas during the 1943 season. The minor elements in wheat and soil did not show such a definite trend across the state as the major elements.

It is well known that the mineral composition of wheat is influenced by such factors as season, locality, and variety. Excellent reviews of the literature on this subject have been given by Sullivan (8), Beeson (3), and Bailey (1). Most of these studies, however, have dealt with relatively small areas and isolated sections of the wheat-growing belt. Most of them also have not been concerned with consecutive crops grown in the same locality. In view of these facts and because of the importance of Kansas as a wheat-producing area, a study was undertaken of the mineral composition of several standard varieties of hard red winter wheat grown in various locations in the state. The results are presented in this report.

Materials and Methods

Design of the Experiment. The study was extended over a three-year growing period, with samples obtained from the same plots each

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year. In addition, soil analyses from the same plots were made for two years and average values are presented in order to correlate these data with the grain analyses. Other data presented include average rainfall, protein, ash, yield, and test weight. The data taken as a unit, therefore, combine many of the factors generally considered to have an influence on the growth of wheat, and cover the state of Kansas for wheat grown in 13 different localities, including in each location, samples of Turkey, Blackhull, and Tenmarq. The following report presents the data as obtained and the methods used for the analyses.

Analysis of Wheat Samples. Wheat samples were ground and analyzed for the following constituents: moisture, ash, protein, potassium, calcium, magnesium, phosphorus, sodium, manganese, iron, and copper. For the determination of total ash, the samples were ignited in a muffle at 600°C. for 16 hours. Protein determinations were made by the usual A.O.A.C. Kjeldahl procedure. A factor of 5.7 was used to convert per cent nitrogen to per cent protein.

Potassium was determined colorimetrically by the procedure Harris (4) recommended. Calcium was determined by titration with permanganate. After a 20-mg. sample of ash had been dissolved in the buffer solution, the method was similar to that which Wang (9) used for blood serum.

The method used for magnesium was similar to that described by Lindner (5) which makes use of titan yellow as a colorimetric reagent. This method is satisfactory for plant materials provided the pH is kept constant. It was found desirable to use 3.5 ml. of 15% potassium hydroxide rather than 1 ml. of the 40% solution as recommended by Lindner.

The remaining mineral constituents, phosphorus, sodium, manganese, iron, and copper, were determined spectrographically on a Bausch and Lomb large litrow spectrograph, using the standard solutions, spectral lines, and techniques suggested by Morris, Pascoe, and Alexander (7), with the following changes which were necessary principally because of different spectrographic equipment. Solutions of the samples were placed on electrodes as recommended by Morris *et al.* (7). The image was focused on the collimating lens and the electrode spacing was set at 1 mm. The samples were arced for 5 minutes. The excitation source was a high voltage A.C. arc at 2200 volts and 2.4 amperes. The sector was set at three-eighths open. All spectra were taken in duplicate and a set of previously prepared standards was placed on each plate. Plates were developed as recommended by Morris.

Analyses were made by use of the spectral lines recommended by Morris, and line densities were read with an A.R.L.-Dietert densitom-

eter. Per cent composition was then determined from standard curves. Cadmium was used as an internal standard.

Analysis of Soil Samples. Soil samples, obtained from the same fields which produced the grain, were analyzed for available potassium, calcium, magnesium, nitrogen, manganese, iron, copper, and phosphorus.

The soil samples were extracted with Morgan's solution (6) and the extracts analyzed for the readily available plant nutrients which were present. The procedures used for nitrogen, potassium, magnesium, phosphorus, and calcium were essentially those presented by Wolfe (10, 11) and later summarized by him (12).

The remaining constituents, manganese, iron, and copper, were determined spectrographically. The method was essentially the same as with plant material. However, a few changes were required due to the presence of the salts used in Morgan's extracting solution. Standards were prepared in the presence of the extracting solution for comparison purposes, because of the effect of large quantities of salts on the line intensities of the minor elements. Spectrographic plates were taken under the conditions previously described and concentrations calculated in the same manner. As sodium was present in the extracting solution it was not determined in the soil.

Results and Discussion

An outline map of Kansas showing the location of the test plots under the supervision of the agronomy department is shown in Fig. 1. The samples represent widely scattered localities and quite different growing conditions. The wheat samples were secured from the same plots all three years. During the 1944 season no samples were available from Tribune or Smith Center.

Table I presents data of the United States Weather Bureau for the average rainfall at each location. The variations in rainfall, in conjunction with differences in basic soil types, could be responsible for quite different results at different locations. Rainfall at the various locations was average or slightly higher during the period of the survey.

Table I also gives the average yield and test weight at each location. Good yields were obtained in most instances. In 1943, however, yields at Meade and Dodge City averaged only 5.0 and 7.4 bushels per acre respectively. Hays produced 9.5 and Belleville 8.1 the same year. These locations produced the lower average yields for the three-year period as shown in the table. Average yields for all plots sampled were respectively 20.6, 30.0, and 33.4 bushels per acre during the three-year period with an overall average of 28.0 bushels

per acre for the survey. Test weight values varied somewhat with location, with the Tribune, Kingman, and Wichita fields showing the highest averages. The Blackhull variety exhibited a slightly higher test weight than the two other varieties studied.

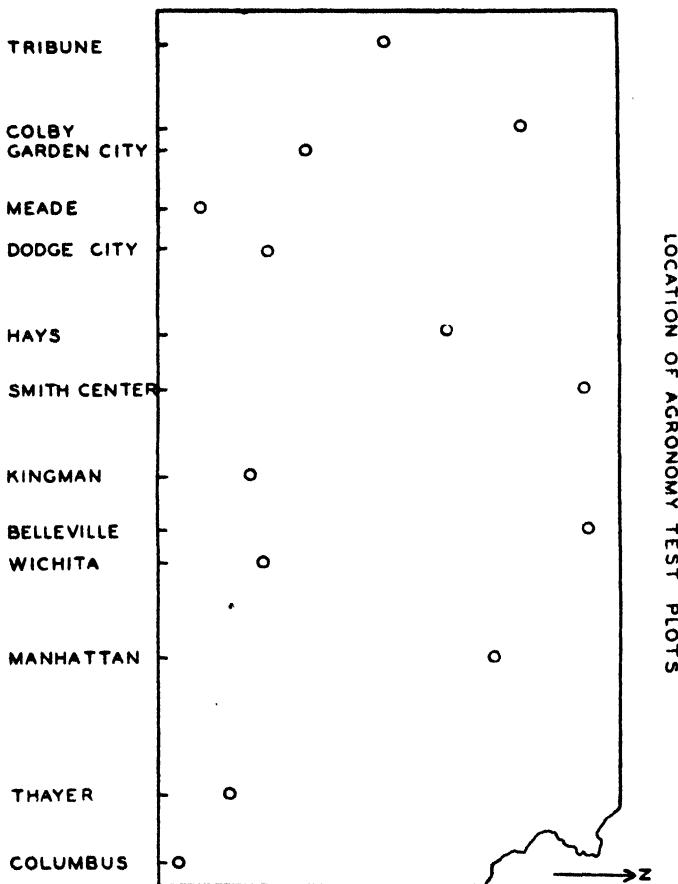


Fig. 1. Outline map of Kansas showing locations from which wheat samples were obtained.

Ash and Protein Content. Table II presents data regarding average ash and protein content of the wheat samples. The variation in total ash, or mineral content, at the various locations is striking; in fact, location was more important than other conditions in determining ash content. High ash samples usually occurred at the same location each year. The highest values for mineral content were obtained from the southwestern and central areas of the state, i.e., at Meade and Dodge City and at Hays. Furthermore, these areas were

TABLE I

MEAN RAINFALL, YIELD, AND TEST WEIGHT OF WHEAT GROWN AT
VARIOUS LOCATIONS IN KANSAS
(Averages for 1943-1945 inclusive)

Location	Average annual rainfall <i>Inches</i>	Yield per acre <i>Bu.</i>	Test weight <i>lb./bu.</i>
Tribune	15.8	40.9	60.4
Colby	17.7	48.9	59.4
Garden City	18.5	29.1	57.9
Meade	20.4	22.4	56.2
Dodge City	19.8	21.6	56.9
Hays	22.6	18.1	56.4
Smith Center	21.9	27.7	58.4
Kingman	29.5	22.8	61.1
Belleville	26.2	19.3	53.5
Wichita	29.7	28.1	60.3
Manhattan	31.0	26.9	58.3
Thayer	39.9	32.2	57.9
Columbus	41.8	31.2	57.0

consistently high all three years. There were no consistent varietal differences apparent, location being the more important factor.

The protein content of the wheat varied in a somewhat similar manner, although a general trend from one year to the next was observed. In 1943 protein content was highest, with somewhat lower levels in 1944 and 1945. Areas which produced high mineral content also produced wheat of high protein content; the top five locations with regard to ash content included the top four locations in protein.

TABLE II

MEAN ASH AND PROTEIN CONTENT OF WHEAT GROWN AT
DIFFERENT LOCATIONS¹
(Mean values for 1943-1945 inclusive)

Location	Ash %	Protein %
Tribune	1.86	14.8
Colby	1.92	15.0
Garden City	2.09	16.9
Meade	2.43	17.2
Dodge City	2.36	16.3
Hays	2.33	17.7
Smith Center	1.93	16.7
Kingman	1.82	13.5
Belleville	2.33	13.0
Wichita	1.91	14.0
Manhattan	2.03	13.6
Thayer	1.91	12.4
Columbus	2.00	11.7

¹ All data on this and the following tables are on a moisture-free basis.

The fifth location, which consistently produced wheat of high mineral content but of lower protein, was the Belleville field. As would be expected, the areas which received the most rainfall produced good yields of slightly below average ash content and low protein. Kingman, with the lowest ash content, also produced wheat of lower protein content.

Major Mineral Elements. Table III contains data on the four metallic elements found in greatest amount in wheat—potassium, phosphorus, magnesium, and calcium. The oxides of these four elements account for well over 90% of the total ash. Wheat samples obtained from western fields averaged higher in mineral content in all cases, presumably due to the higher levels of available nutrients in the soil.

TABLE III

MEAN POTASSIUM, PHOSPHORUS, MAGNESIUM, AND CALCIUM CONTENT IN
THREE VARIETIES OF KANSAS WHEAT GROWN AT VARIOUS LOCATIONS
(Means for 1943-1945 inclusive)

Location	K	P	Mg	Ca
Tribune	0.44	0.34	0.133	0.051
Colby	0.41	0.36	0.139	0.048
Garden City	0.40	0.42	0.152	0.051
Meade	0.43	0.53	0.157	0.049
Dodge City	0.45	0.50	0.153	0.057
Hays	0.45	0.41	0.142	0.066
Smith Center	0.40	0.38	0.122	0.045
Kingman	0.37	0.34	0.104	0.036
Belleville	0.42	0.50	0.136	0.047
Wichita	0.38	0.39	0.109	0.038
Manhattan	0.36	0.44	0.131	0.035
Thayer	0.35	0.47	0.116	0.041
Columbus	0.36	0.53	0.129	0.036

Potassium averaged highest in the samples obtained from the Dodge City, Meade, and Hays fields. These fields are also among those high in available potassium, as shown in the soil analyses presented in Table V. Likewise, the lowest potassium levels in the grain were from fields of lowest potassium content in the soil.

Phosphorus data show an interesting deviation from the correlation with the soil data. The samples obtained from the Columbus and Thayer fields were taken from plots which had been fertilized with additional phosphate. The general downward trend in phosphorus as the eastern border of the state was approached was reversed at these locations, due undoubtedly to the added fertilizer these plots received. Unfortunately, samples of wheat grown on adjacent untreated plots were not available for comparison. Samples from

the Belleville field in north central Kansas also contained larger amounts of phosphorus than other fields of about the same level of available phosphorus in the soil.

Magnesium in the grain also averaged higher in the western samples. The lowest magnesium content in grain was found in samples from Kingman, which also had the lowest magnesium content in the soil. The general trend in magnesium is not quite so evident as were those of potassium and phosphorus.

Calcium also follows the generalization that western Kansas grains are higher in the inorganic constituents of the ash, although the highest average calcium in the grain was not produced on the test plots of highest calcium. The Hays test plots produced wheat of the highest calcium content every year. Wheat produced at Hays had the highest average protein content. Other western Kansas test plots also produced wheat of higher calcium content than those located in eastern Kansas.

The higher mineral content of the western Kansas wheats is due not to any one element, but rather to the combined effect of the several major constituents of the ash. The variation in mineral content of the grain is much less than the variation of the corresponding element in the soil. For example, there was an eightfold variation in available calcium in the soil and only a twofold variation in the calcium content of the grain.

Minor Mineral Elements. Table IV contains data regarding the minor elements (manganese, iron, copper, and sodium) included in the study. Although a number of other elements have been shown by Sullivan (8) to be constituents of wheat, they have not, as yet, been quantitatively determined in this laboratory.

These minor elements vary more than the major constituents of the ash. In several cases the average figures in Table IV do not give an indication of yearly variability. In 1943 wheat grown at Meade and Dodge City contained about four times as much manganese as that grown farther east. This trend was not particularly noticeable the following two years. Since it is known that the availability of manganese is influenced greatly by soil moisture, it may be assumed that more optimal conditions for absorption of manganese occurred in this area in 1943 during the proper stage in plant growth to produce a grain high in manganese; iron, also, showed a somewhat similar trend, being higher in the western area in 1943 than in the two following years.

Sodium content of the grain was quite uniform throughout the state all three years. It was slightly lower in 1945 than the previous two years. In general, the sodium content of Kansas wheat was some-

what higher than values reported by Morris *et al.* (7) and Sullivan (8) on their samples.

Copper is known to be essential in plant growth. Barham *et al.* (2) have recently suggested its possible correlation with the starch content of the sorghum grains. This study seems to indicate that the areas included in this survey contain sufficient copper for proper plant growth. The copper content of the wheat, although varying somewhat, was generally uniform throughout the state.

TABLE IV
MEAN SODIUM, IRON, MANGANESE, AND COPPER CONTENT IN THREE
VARIETIES OF KANSAS WHEAT GROWN AT VARIOUS LOCATIONS
(Crop years 1943-1945 inclusive)

Location	Na	Fe $\times 10^3$	Mn $\times 10^3$	Cu $\times 10^3$
	%	%	%	%
Tribune	0.016	0.66	0.55	0.69
Colby	0.015	0.79	0.57	0.55
Garden City	0.020	0.85	0.70	0.65
Meade	0.022	1.21	0.81	0.77
Dodge City	0.020	1.00	0.55	0.74
Hays	0.019	0.70	0.38	0.65
Smith Center	0.014	0.61	0.37	0.43
Kingman	0.014	0.50	0.26	0.53
Belleville	0.018	0.70	0.49	0.71
Wichita	0.014	0.53	0.48	0.67
Manhattan	0.015	0.67	0.29	0.55
Thayer	0.016	0.65	0.42	0.64
Columbus	0.017	0.70	0.43	0.70

Available Nutrients in Soil. A definite correlation exists between available nutrients (Table V and VI) and the wheat analyses. The soil samples were obtained in 1943 and 1944 from the same plots on which the wheat was grown. In general, the trend in readily available nutrients follows an order dependent on rainfall. Those areas which have received more rainfall are those in which nutrient materials have been leached out of the soil.²

Soil data indicate that the major nutrients are still in good supply in Kansas except in the extreme southeast. This area receives the greatest rainfall and has been under cultivation longer than other areas of the state. This section of the state produces wheat of lower protein content, but it is not a part of the main wheat-producing belt of the state. The soils of the extreme southeastern part of the state show favorable response to the additions of commercial fertilizers.

The data presented in Table V show that there is a decided variation in the readily available potassium, calcium, phosphorus, and nitro-

² The general classification of the soil in each location may be readily obtained from the map of Kansas entitled "Natural Agricultural Resource Areas of Kansas" compiled for Region 5, Soil Conservation Service, by C. L. Fly, soil scientist.

TABLE V

MEAN AVAILABLE POTASSIUM, CALCIUM, PHOSPHORUS, AND NITROGEN
CONTENT, AND pH IN KANSAS SOIL¹ FROM VARIOUS LOCATIONS
(Crop years 1943-1944 inclusive)

Location	pH	Parts per million			
		K	Ca	P	N
Tribune	6.70	222	9400	51.7	100
Colby	6.58	265	4580	49.8	115
Garden City	7.04	146	8600	47.1	98
Meade	6.65	253	3370	40.3	117
Dodge City	6.28	204	4060	42.1	109
Hays	6.59	191	3300	48.8	71
Smith Center	6.17	181	3040	37.8	77
Kingman	5.95	78	1960	26.0	86
Belleville	6.18	118	2830	27.4	100
Wichita	6.23	148	2730	30.2	124
Manhattan	6.61	114	2980	23.7	91
Thayer	6.08	25	1940	18.1	64
Columbus	6.00	23	1140	14.8	67

¹ 0-20 inches.

gen content of the soils as well as in the pH of the soil solutions in the various localities. For example, potassium varies from over 250 ppm. in the west to 23 ppm. in the southeast. Calcium shows similar variations. pH measurements also indicate variations in the presence of the base-producing ions, with a range in pH values of from 7.04 to 5.95. The areas of high nutrient availability coincide with those producing the wheat of highest ash and protein content.

TABLE VI

MEAN AVAILABLE MAGNESIUM, MANGANESE, IRON, AND COPPER
IN KANSAS SOIL¹ FROM VARIOUS LOCATIONS
(Years 1943-1944 inclusive)

Location	Parts per million			
	Mg	Mn	Fe	Cu
Tribune	172	35	36	3.0
Colby	144	43	31	3.1
Garden City	190	25	33	3.2
Meade	154	69	35	3.3
Dodge City	150	61	45	3.3
Hays	262	38	27	2.7
Smith Center	252	40	31	3.1
Kingman	59	47	32	2.4
Belleville	110	45	26	3.1
Wichita	148	49	41	3.2
Manhattan	214	62	29	3.5
Thayer	117	65	41	3.7
Columbus	123	42	40	3.4

¹ 0-20 inches.

The data on soil samples from the Kingman field offer an opportunity for an interesting evaluation of soil and grain analyses. Kingman is relatively low in all the nutrients for which analyses were made, although not the lowest in every case. The grain from this area consistently had the lowest ash content, and protein was also quite low. This would indicate an effect due to the decreased availability of the required elements even though they may be present in amounts which may be above the minimum generally recognized as required for the crops. Interestingly enough, the wheat grown at the Kingman field had the highest test weight during the period under study. The decreased availability of the necessary elements is also noticed in Table VI. For example, magnesium is very low in comparison to other fields. The other minor elements which were included in the survey were also well below average. The lowered availability of the elements in this area is reflected in the generally decreased quantity of each of the elements in the grain as indicated in Tables III and IV.

The data presented in Table VI, which include magnesium, manganese, iron, and copper, do not show entirely the same general and definite trend as was the case with the elements included in Table V. The copper content of the soil is fairly uniform in the fields sampled, although in the central area it is somewhat lower than in the others. Magnesium is higher in the west, and as noted previously is lowest in the samples taken from the Kingman field. Manganese varies considerably in different localities; although the manganese was very high in samples of grain from the Meade and Dodge City fields in 1943, several other fields were almost as high in the manganese content of the soil. Iron varied somewhat over the state, also, but the trend was not regular. The central area was lower in available iron, and the samples from Thayer and Columbus contained more iron, in general, than did those from other areas.

Acknowledgments

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AMYLOSE CONTENT OF INDIAN CORN STARCHES FROM NORTH, CENTRAL, AND SOUTH AMERICAN CORNS¹

ROY L. WHISTLER² and PAUL WEATHERWAX³

ABSTRACT

Analyses of starch samples from 39 different Indian corns indicate iodine adsorption values of 44.4-56.6 mg. per gram of starch or amylose contents of 22.2-28.3%, the average being 24-25%. Thus, these starches have usual amylose contents.

Corn starches which have previously been analyzed for amylose content may be classified into two principal groups: (A) those from waxy or low amylose corn varieties which contain 0-6% amylose, and (B) those from practically all other corn varieties, including both open-pollinated and hybrid varieties of dents, pop, and sweet corns which contain 25-29% amylose. Only a few waxy or low amylose starches are known. Recently a sugary mutant (3) has been found to contain 50-65% amylose and hence cannot be classified in either group. All other corn starches on which published data are available fall into the second group.

Recently there have become available a number of corn varieties from widely different localities ranging from Arizona and New Mexico to southern South America. For the most part, these samples represent relatively unimproved varieties which have been grown by the Indians for centuries. They include a variety of different pigmenta-

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tions and kernel sizes and shapes. Endosperm types range from horny to mixtures of horny and floury and to almost entirely floury. It was of interest, therefore, to analyze the starches from a number of these samples to determine whether any of them might contain unusual amounts of amylose.

Materials and Methods

Corn Samples. Samples of the different varieties of corn were collected by one of the authors (Weatherwax) during the course of a survey which extended from New Mexico and Arizona to South America. Funds for the survey were made available through the generosity of the Guggenheim Foundation, Indiana University, and the Funk Brothers Seed Company. Sources of the various samples are shown in Table 1. Sample ears were obtained from the native farms or fields or from native markets.

Preparation of Starch. Four or five representative kernels from each ear were selected, the grain soaked in water for 2 or 3 hours and the pericarp of each removed with a knife. The aleurone was also scraped away as completely as possible. After cutting out the germ, the remainder of the kernel was ground to pass a 60-mesh screen by a laboratory Wiley mill. In one instance, however, in order to make a comparison of the horny and floury endosperms, the two were separated by first grinding out the floury part by means of a dental drill. The residual horny portion was then ground in a Wiley mill. Samples of powdered endosperm varied in weight from 0.2 to 1.5 g.

Fat was removed from the powdered endosperm by four successive one-hour extractions with refluxing 85% methanol (6, 7). Approximately 10 ml. of methanol was used for each reflux period, and the mixture was stirred briefly at frequent intervals. After each reflux period, the sample was filtered on a microfunnel, washed well with methanol, and then mixed with 10 ml. of fresh methanol. After the final extraction, the crude starch was filtered, washed with methanol, and air-dried.

Analytical Methods. Since the quantity of material in each sample was small, moisture determinations were made by the following indirect procedure: A number of samples were spread out in thin layers and allowed to equilibrate with the atmosphere along with a 6-8 g. sample of commercial corn starch previously methanol-extracted in the same manner as the small samples. At the end of four days, triplicate moisture determinations were made on the large sample and its moisture content was assumed to represent that of the small samples.

Amylose was determined by a method only slightly different from

that described by Bates, French, and Rundle (1) and modified by Wilson, Schoch, and Hudson (8). Starch samples were dissolved in 0.5 *N* potassium hydroxide by allowing the mixture to stand for approximately 24-48 hours at 0°C. in an atmosphere of nitrogen. After the solution of the starch, a small amount of undissolved protein and possibly some cellulosic fiber remained in the solution. Since this material, which constituted less than 1% of the sample, was found not to affect the result of the subsequent potentiometric iodine determination, it was allowed to remain in the solution. Iodine values were corrected for free iodine and hence represent the total number of milligrams of iodine bound per gram of starch.

Sources of Error. The method of indirect moisture determination leads to little, if any, error. This was shown in several instances by determining moisture on the micro samples as well as on the large samples. In all the tests made, moisture values of the small samples were identical with those of the large samples.

Both the protein and ash in the endosperm serve as inactive diluents in the starch. The ash content in the endosperm of several varieties of commercial corn has been shown (4) to vary from 0.22 to 0.46%. Similar ash contents were assumed for the endosperms examined in this investigation. These values were below the limits of error for the survey method used here, and hence the ash content of the endosperm is disregarded in the subsequent calculations of amylose content.

The greatest error encountered is that involved in the estimation of protein. In an examination of the protein content of the endosperm from 11 varieties of commercial corn, Earle, Curtis, and Hubbard (4) found a variation of 6.7 to 12.8% with an average of 9.4%. Hansen, Brimhall, and Sprague (5), in examining a wide variety of endosperm types, noted protein contents which varied about 5 to about 22% with an average of approximately 12%. The results show that roughly half of the protein is extractable with alcohol. This soluble protein would be removed during the defatting treatment described above. Consequently, it was assumed for the purpose of calculating amylose contents that the methanol-extracted starch herein investigated contained approximately 5% protein. Potentiometric iodine values were correspondingly corrected. These assumptions would indicate an analytical error of about $\pm 5\%$.

A test of this procedure was obtained by applying it to the analysis of a sample of corn whose starch is known to have an iodine sorbing value of 51.7 mg. per gram. A value of 51.0 was obtained for the starch by this procedure. Amylose content in per cent may be calculated by assuming that pure amylose sorbs 200 mg. of iodine per gram (unpublished data of J. A. Wolff and R. L. Whistler).

In several instances where sufficient grain was available, the starch was isolated and purified by the method of Brimhall, Sprague, and Sass (2). Analytical values of the starch indicated the same amylose contents as found with the microtechnique described above.

Results and Discussion

Analyses of starch samples from 39 different Indian corns indicate iodine sorption values (Table I) of 44.4 to 56.6 mg. per gram of starch

TABLE I
AMYLOSE CONTENT OF INDIAN CORNS

Sources	Iodine affinity mg./g.
San Juan, Pueblo, New Mexico, 1819.12 ¹	50.6
Retalhuleu, Guatemala, 1849.4	52.6
San Juan, New Mexico, 1819.26	52.0
San Juan, New Mexico, 1819.16	46.8
Huancayo, Peru, 1872.66	45.8
Huancayo, Peru, 1872.103	51.4
Huancayo, Peru, 1872.134	51.6
Navajo, Ft. Defiance, Arizona, 1877.19	48.2
Navajo, Ganado, Arizona, 1875.12	51.0
Navajo, Ganado, Arizona, 1875.17	48.4
Arequipa, Peru, 1860.4	50.8
Arequipa, Peru, 1860.6	48.0
Chimaltenango, Guatemala, 1842.3	45.6
Antigua, Guatemala, 1846.1	48.6
Calca, Peru, 1864.3	50.2
Chacan, Peru, 1865.17	46.2
Hopi, Moenkopi, Arizona, 1831.10	51.0
Huancayo, Peru, 1872.82a	47.2
Jemez, Pueblo, New Mexico, 1823.3	49.6
Quetzaltenango, Guatemala, 1845.5	45.6
Quito, Ecuador, 1857.8	48.6
Paguate Pueblo, New Mexico, 1824.9	50.4
Paguate Pueblo, New Mexico, 1824.13	48.4
San Idelfonso Pueblo, New Mexico, 1818.3	49.6
Calca, Peru, 1864.15	47.2
Santa Ana Pueblo, New Mexico, 1822.5	50.2
Santa Clara Pueblo, New Mexico, 1820.5	55.0
Santa Clara Pueblo, New Mexico, 1920.15	51.8
Santa Clara Pueblo, New Mexico, 1920.21	56.6
Santo Domingo, Ecuador, 1859.2	53.4
Santo Domingo, Ecuador, 1859.10	48.8
Tecpan, Guatemala, 1843.3	46.6
Tesuque Pueblo, New Mexico, 1817.2	54.2
Tesuque Pueblo, New Mexico, 1817.3	48.2
Tesuque Pueblo, New Mexico, 1817.5	45.0
Toluca, Mexico, 1834.16	49.4
Toluca, Mexico, 1834.17	44.4
Toluca, Mexico, soft white, 1834.18	51.2
Zufi Pueblo, New Mexico, 1829.3	47.0
Indiana Hybrid 644, floury endosperm	49.6
Indiana Hybrid 644, horny endosperm	48.6

¹ Numbers are those identifying the sample in the collection of Paul Weatherwax.

or amylose contents of 22.2 to 28.3%, the average being 24-25%. This compares with an amylose content of 26% (iodine sorption value of 51.7) for seven starches from standard corn belt corns.

This uniformity of composition occurs despite the fact that the corn samples were obtained from widely separated areas and differed considerably in their appearance, coloration, and relative amounts of floury to horny endosperm.

Although the analytical values obtained in this survey may be in slight error, they clearly do not indicate the presence of waxy starches or starches with exceptionally high amylose contents.

Analyses of the separated horny and floury endosperms of a standard corn belt corn (Indiana Hybrid 644) indicate about the same amylose content for both types of starch.

Acknowledgment

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Abstract. A concise abstract of about 200 words follows title and authors. It should state the principal results and conclusions and should contain, largely by inference, adequate information on the scope and design of the investigation.

Literature. Long introductory reviews should be avoided, especially when a recent review in a monograph or another paper can be cited instead. References are arranged and numbered in alphabetical order of authors' names and show author, title, journal, volume, first and last pages, and year. The list is given at the end of the paper. Reference numbers must invariably be cited in the text, but authors' names and year may be cited also.

Organization. The standard organization involves main sections for abstract, introduction, materials, methods, results, discussion, acknowledgments, and literature cited. Alternately, a series of separate studies is often best described with main sections for each study. Headings should be restricted to center headings for main sections and run-in italicized headings for subsections.

Tables. Data should be condensed and arranged to facilitate the comparisons the reader must make. Tables should be kept reasonably small by breaking up large ones, omitting all unessential data, and minimizing number of significant figures. Leader tables without number, main heading, or ruled lines are useful for small groups of data. Textual matter in tables should be minimized and unnecessary footnotes should be avoided.

Tables should be typed on separate pages at the end of the manuscript and their positions should be indicated to the printer by typing "(Table I)" in the appropriate place between lines of the text. (Figures are treated similarly.)

Figures. If possible, all drawings should be made by a competent draughtsman. Curves should be drawn heaviest, axes or frame intermediate, and grid lines lightest. The horizontal axis should be used for the independent variable; and experimental points should be shown. Labels are preferable to legends. All drawings should be made two to three times eventual reduced size with India ink on white paper, tracing linen, or blue-lined graph paper. Lettering should be done with a guide, and letters should be $\frac{1}{8}$ to $\frac{1}{4}$ inch high after reduction.

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HYDROLYTIC TREATMENT OF SOYBEAN PROTEIN WITH PAPAIN¹

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ABSTRACT

A study has been made of the effect of papain on the solubility of soybean protein determined at its isoelectric point. The substrate system was the water extract of soybean meal. While the pH of maximum activity was found to be about 8.0, there was good activity in the region of pH 5.0 to 9.0. Proteolysis proceeded rapidly without the use of activators. Hydrogen peroxide, sodium peroxide, and sodium hypochlorite were good deactivators for the system. Ninety-five per cent of the protein became water soluble at pH 4.2 in 3 hours at 60°C.

There are a number of food and industrial applications for soybean protein where various degrees of hydrolytic treatment are desirable, either for modifying the physical properties of the dispersed protein or for increasing its dispersibility in mild alkalies or water.

An outstanding example of protein modification by hydrolytic treatment is in the development by Urquart (12) and Perri (5, 6) of a foam stabilizer which served such a useful purpose during the war.

Smith and Max (11) studied the adhesive properties of soybean protein as they are affected by a mild hydrolytic treatment. They found that a protein prepared by simple alkaline extraction and acid precipitation has a low adhesive value if dispersed in alkaline salts, such as sodium carbonate or trisodium phosphate, but has a high adhesive value if dispersed in sodium hydroxide. In contrast to this behavior, the adhesive value of the protein in alkaline salts is greatly increased if the protein has received a mild hydrolytic treatment with sodium hydroxide during its preparation. This simple experiment illustrates a fundamental difference between soybean protein and casein, since the adhesive value for casein is not significantly different when dispersed in either mild or strong alkalies.

There are other applications where modification in dispersion characteristics resulting from mild hydrolytic treatment would be

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expected to improve the product. Such applications include textile sizing, paper sizing, water emulsion paints, whipping agent, vegetable milk, and its use as a protective colloid.

If the desired hydrolytic treatment with alkali or with acid is at room temperature, it requires either considerable reagent or much time. Conducting the hydrolytic treatment at elevated temperatures would shorten the time factor but would bring other difficulties into operation, especially control of the reaction. The present investigation was undertaken to explore the possibilities of shortening this time factor by carrying out a mild hydrolytic treatment with an enzyme. The enzyme papain, described by Balls, Thompson, and Jones (1), was selected because it is a strong proteinase, relatively abundant, and relatively cheap. The present investigation is limited to a survey of the factors known to have the greatest influence on proteolytic action, such as pH, temperature, concentration of the enzyme, and the use of activators and deactivators. For the enzymatic process to be commercially feasible, it must be precisely controlled, and a method must be available for complete deactivation at a given stage of the reaction.

Materials and Methods

The protein system used in this investigation was a water extract of hexane-extracted soybean meal and contained, along with the protein, the sugars, phosphatides, salts, and other water-soluble constituents of the soybean meal. The protein in the system constituted less than half of the total solubles. The water extract of the meal had a pH of 6.6 to 6.7. It was anticipated that the behavior of such a system might be different from that of a solution containing only protein. The solution was prepared by stirring flakes with 12 times their weight of water and then removing the undissolved meal in a centrifuge. Such a dispersion contained about 1.8% protein of which 79.5% was precipitable with acid at pH 4.2.

The enzyme used was a water extract of commercial whole Ceylon papain. In making up the enzyme solution, the crude papain was ground in a mortar with a small amount of water, then diluted to 40 parts of water, mechanically shaken for 20 minutes, and centrifuged to remove the insoluble residue. The enzyme solution was further clarified by filtration. It had a pH value of 5.3.

Several different samples of crude papain were analyzed and found to contain 9.6% to 10.1% nitrogen, of which 94% to 96% was dispersible in water at a ratio of 1 to 40. However, one batch of enzyme was used for nearly all the work reported here. The reproducibility of the method of preparing the enzyme was found to be satisfactory by comparing the activity of several samples. The activity of the enzyme

was checked at intervals by measuring its action on gelatin by the Van Slyke amino nitrogen method. The experimental points on the curves in Figs. 1 to 5 are the average results of two or more experiments. The data in Tables I and II are the results of a single experiment.

Method of Measuring Hydrolysis. The measurement of hydrolysis of soybean protein in the volumetric Van Slyke apparatus was impractical because of the insolubility of the protein under the conditions of measurement. Other methods were tried and the one found to be the most satisfactory gave the change in the protein solubility at its isoelectric point of pH 4.2 as determined by Smith and Circle (8, 9) and Circle and Smith (2) with proteolysis. After a given reaction period, the enzyme was inactivated, the pH adjusted to 4.2, the dispersion centrifuged, and the nitrogen in the centrifugate determined. The increase in soluble nitrogen over that in the unhydrolyzed protein was taken as a measure of hydrolysis. The change in solubility of the protein with enzyme hydrolysis was much greater in the early than in the later stage of the reaction. However, for the present study the early stage is the more important part of the reaction and by any method of measurement the results are only relative.

Results

Effect of pH on Papain Activity. Hoover and Kokes (4) recently studied the effect of pH upon proteolysis of casein by papain. From their work and that of others it is evident the effect of pH on enzyme activity varies somewhat with the substrate. It was necessary, therefore, to survey the effect of pH on the soybean protein system and to find the region of good enzyme activity as well as the regions of inactivity. It was considered possible that the reaction could be controlled, in practical application, through pH adjustments.

A problem arising from the "change in solubility" method, which requires special consideration in the measurement of effect of the pH on activity, is the influence of buffer salts for maintaining constant pH on the dispersibility of the protein. The present work is directed toward a practical application where the use of added buffers could not be economically justified. Fortunately, these added buffers have been unnecessary for satisfactory pH control. At 25°C. and without buffers, the greatest pH change (lowering) of the system occurred in the region of pH 4.0 to 8.0 and for the first 2 hours amounted to less than 0.2 pH units. A pH lowering, superimposed on that of the enzyme action, in the range of 6.5 to 8.5, and at about 40°C., is caused by the action of microorganisms on sugars. Without addition of enzymes this change may amount to 1.5 to 2.0 pH units in 20 hours. Smith and

Max (10) isolated protein from similar systems after they had been subjected to the souring action of bacteria and yeast for 20 hours at about 40°C. They found no appreciable difference in the amount of nitrogen precipitated by acids before and after the action of the souring microorganisms. When the reaction is carried out at 26°C. for a short period the effect of bacteria and enzyme on pH is greatly minimized. It will also be noted from the pH activity curve (Fig. 1) that activity does not vary greatly in the range of 6.0 to 8.0, thereby further minimizing the need for precise buffer control to obtain practical results in this region. At pH values of 11.0 and 1.7, the inactive regions for the enzyme system as well as little bacterial action, the pH change, without buffers, was no more than 0.2 unit for 20 hours.

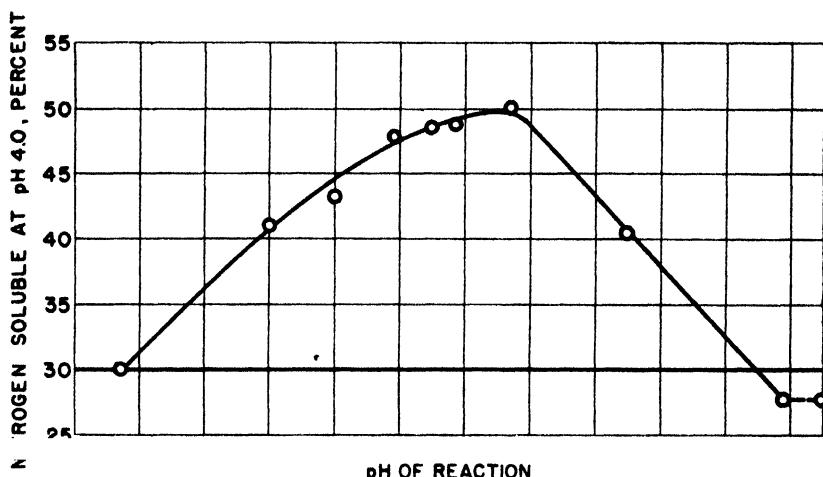


Fig. 1. The effect of pH on the hydrolysis of soybean protein with papain at 26°C. and E:S nitrogen ratio of 1:15. Reaction time 1 hour.

The salts added to the system (sodium sulfate) during neutralization procedures, as well as buffer salts, cause an increase in the protein dispersed; therefore, the salt concentration of the system was kept constant by adding salt solution as necessary. The total salt added amounted to 1.2 g. per 100 ml. solution. In the pH activity data, no correction has been made for the added nitrogen of the crude enzyme. These two factors, added salt and enzyme, give a constant increase of about 9.5% of soluble nitrogen in the system.

The results of the effect of pH on enzyme activity, for a one-hour period, are shown in Fig. 1. For these data the nitrogen ratio of crude enzyme to substrate was 1 to 15. The recorded pH values on this curve are the average of readings taken a few minutes after the addition of the enzyme and again at the end of the one-hour period.

From the results shown in Fig. 1, it may be concluded that the maximum activity is at about pH 8.0; nevertheless, there is good activity throughout the range of 5.0 to 9.0. Separate experiments demonstrated that the enzyme was undergoing inactivation at pH 1.7 and lost activity beyond recovery in 65 minutes at 25°C.

When the enzyme solution (pH 5.3) was added to the water extract of the meal (pH 6.6) a light precipitate was formed, possibly a complex between the protein and enzyme due to widely different isoelectric values. This precipitate disappeared when salts were added or the pH of the system was raised to about 7.5. If the pH of the system

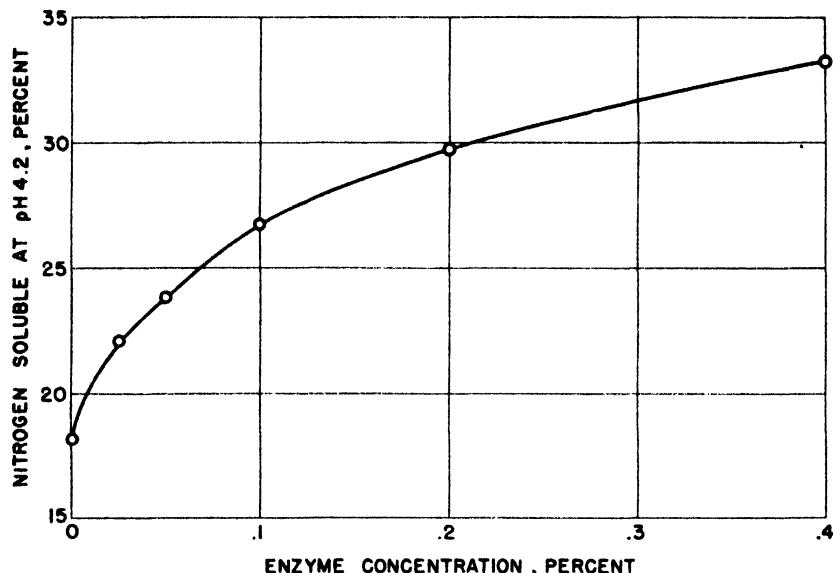


Fig. 2. The relation of concentration of crude enzyme to the change in nitrogen solubility at pH 4.2. Reaction period of 60 minutes, at 26°C., and pH 8. Enzyme concentration based on total weight of solution.

was lowered below 6.6, the precipitate increased. The formation of this precipitate is believed not to be a pH effect on the soybean protein dispersion, as without added enzyme the dispersion shows no precipitation until the pH is lowered below 5.5. It appears probable that the precipitate formed on addition of the papain to the protein is a factor, along with the pH, in reducing enzyme activity in the region below pH 8.0.

Enzyme Concentration. The studies on pH activity were carried out at an enzyme-substrate nitrogen ratio of about 1:15. Further studies were conducted to determine the effectiveness of papain hydrolysis at other enzyme concentrations. The proteolysis was carried

out for 60 minutes at 26°C., with a pH value of 8.0 and without added activators. The results are shown in Fig. 2.

While there is no section of the curve showing a strictly direct variation with enzyme concentration, nevertheless such a relationship is closely approximated in the range of 0.025% to 0.1% of enzyme. The remainder of the work included an enzyme concentration of 0.05% on the weight of the dispersion which is equivalent to an enzyme-substrate nitrogen ratio of about 1:60 or proportionately one-fourth of that used in the pH activity studies.

Activators and Deactivators. It was found that activators, which are customarily required for efficient papain activity, had relatively little effect on the proteolysis as it occurred in the water extract of the soybean meal, and the reaction proceeded efficiently without the addition of reducing agents. The effective proteolytic results obtained without added activators should not be unexpected in view of the work of Gottschall (3). In studies on hydrolytic treatment of beef, liver, and near beer, Gottschall found that apparently the activator is supplied by the exposed —SH groups of the protein and that as proteolysis proceeds the rate of reaction increases because of increased concentration of the —SH groups. While no attempt was made to check the concentration of —SH groups in soybean protein systems, the explanation presented by Gottschall concerning the source of activator may be applicable.

According to Scott and Sandstrom (7) the kind and concentration of activator greatly influence the relative activity of the papain, and as activator concentration increases, its effect passes through a maximum.

TABLE I

DATA SHOWING THE ACTIVATING EFFECT OF HYDROGEN SULFIDE AND THIOPHENOL ON PROTEOLYSIS OF SOYBEAN PROTEIN AT 26° AND 40°C. ONE HOUR REACTION TIME, 30 MG. CRUDE PAPAIN PER 75 G. OF PROTEIN DISPERSION AT pH 8.0¹

H ₂ S saturation %	At 26°C.					At 40°C.		
	Total soluble nitrogen %	Increase in sol. nitrogen ² %	Mg. thiophenol per 100 g. of dispersion	Total soluble nitrogen %	Increase in sol. nitrogen ² %	H ₂ S saturation %	Total soluble nitrogen %	Increase in sol. nitrogen ² %
0	24.3 ²	—	0	23.7 ²	—	0	33.3 ²	—
0.01	25.8	1.5	0.08	24.2	0.5	0.01	33.4	0.1
0.12	25.8	1.5	0.80	25.5	1.8	0.12	34.0	0.7
1.20	26.4	2.1	8.00	30.8	7.1	1.20	35.1	1.8
12.0	27.5	3.2	80.00	28.5	4.8	12.00	36.5	3.2
100	31.3	7.0	800.00	31.8	8.1	100.00	39.7	6.4

soluble nitrogen in the original solution was 21.5%.

the amount of soluble nitrogen in the system without added activator.

² a result of the added activator.

In view of their results, the activating effect of hydrogen sulfide and thiophenol was studied at several concentrations. The results of activator studies are shown in Table I. The data in Table I and also in Figs. 1 to 5, inclusive, demonstrate that added activators are unnecessary for a rapid rate of proteolysis for the present system.

It is a fortunate circumstance that activators are unnecessary for the present system, since those normally used are undesirable in a system intended for industrial or food applications. For the two activators investigated there was no indication of the maximum effect demonstrated by Scott and Sandstrom (7).

Hydrogen peroxide, sodium peroxide, and sodium hypochlorite were examined as deactivators. These oxidizing agents were added from 5% solutions to 75 ml. of water extract of the soybean meal. The pH was adjusted to 7.0 and enzyme added at an E:S nitrogen ratio of 1:15. The system was digested at room temperature for 2 hours. The protein precipitated at pH 4.2, and a portion of the whey was analyzed for nitrogen. The results are shown in Table II in terms of percentage of total nitrogen of the solution appearing in the whey.

TABLE II
EFFECT OF DEACTIVATORS ON PAPAIN ACTIVITY

ML. of 5% deactivator	Nitrogen in whey ¹		
	H ₂ O ₂ %	Na ₂ O ₂ %	NaOCl %
0 ²	22.1	21.5	21.8
0.1	37.5	35.3	38.6
0.3	27.6	31.3	41.5
1.0	22.2	25.2	24.1
3.0	23.3	23.9	16.6
5.0	22.8	24.4	21.3

¹ On the basis of total nitrogen in original dispersion.

² Blank determination, no enzyme or deactivator.

From these data it appears that approximately 1 ml. of a 5% solution of deactivator is sufficient to stop the reaction. This amounts to about 1 part deactivator to 4 parts crude enzyme. A more exacting technique would be required to measure precisely the relative effectiveness of deactivators. Hydrogen peroxide is the most satisfactory of these deactivators, since the sodium peroxide produced a very stable foam in the alkaline region and the hypochlorite discolored the isolated protein.

Fig. 3 shows additional data on deactivation with hydrogen peroxide when an excess of the deactivator was used at the same E:S ratio as in Table II and under various temperature conditions. Curve 5

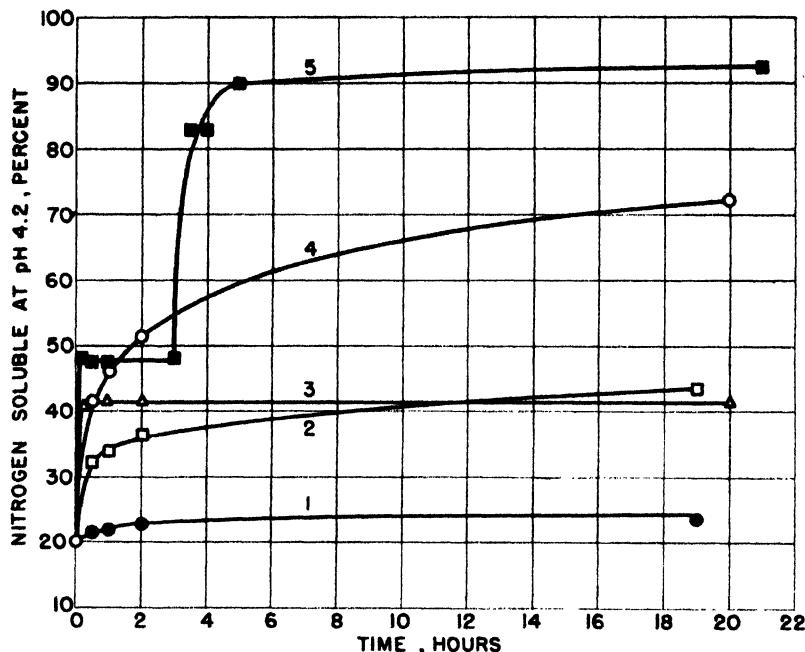


Fig. 3. The effect of hydrogen peroxide as a deactivator at several different temperatures. Curve 1 at 26°C. with hydrogen peroxide added to protein solution before the enzyme. Curve 3 at 40°C. with the hydrogen peroxide added 15 minutes after the enzyme. Curves 2 and 4 are reactions at 26°C. and 40°C. without deactivator. Curve 5 at 60°C. is an example of reversible deactivation with hydrogen peroxide added 5 minutes after the enzyme and sodium sulfite 3 hours later.

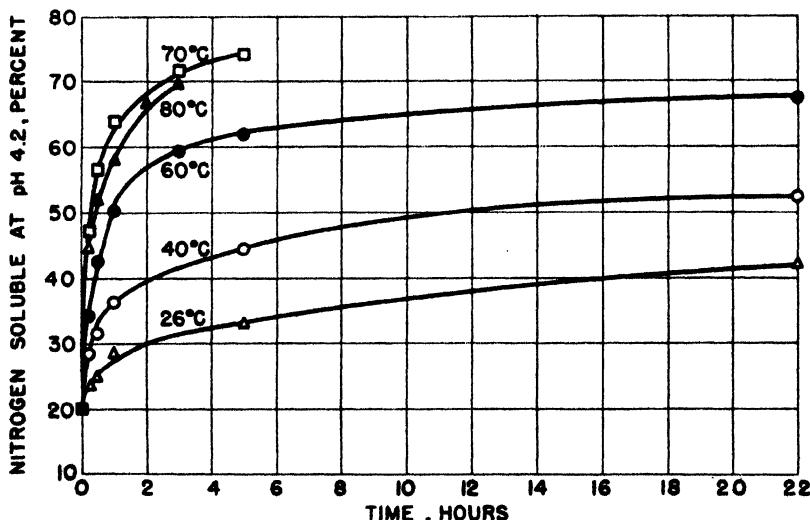


Fig. 4. The effect of temperature on rate of reaction at E:S nitrogen ratio of 1:60 and at pH 8.

of Fig. 3 is an excellent example of reversible deactivation. The hydrogen peroxide was added 5 minutes after the reaction started and brought it to an abrupt halt; 3 hours later the peroxide was destroyed with sodium sulfite and the reaction proceeded at normal or near normal rate as shown by comparing it with curve 5, Fig. 5. This latter reaction is the same as that shown in curve 5, Fig. 3, but without interruption of the hydrogen peroxide and reactivation with the sodium sulfite. When a correction is made for the 3 hours lost due to the hydrogen peroxide, the two curves are very nearly the same.

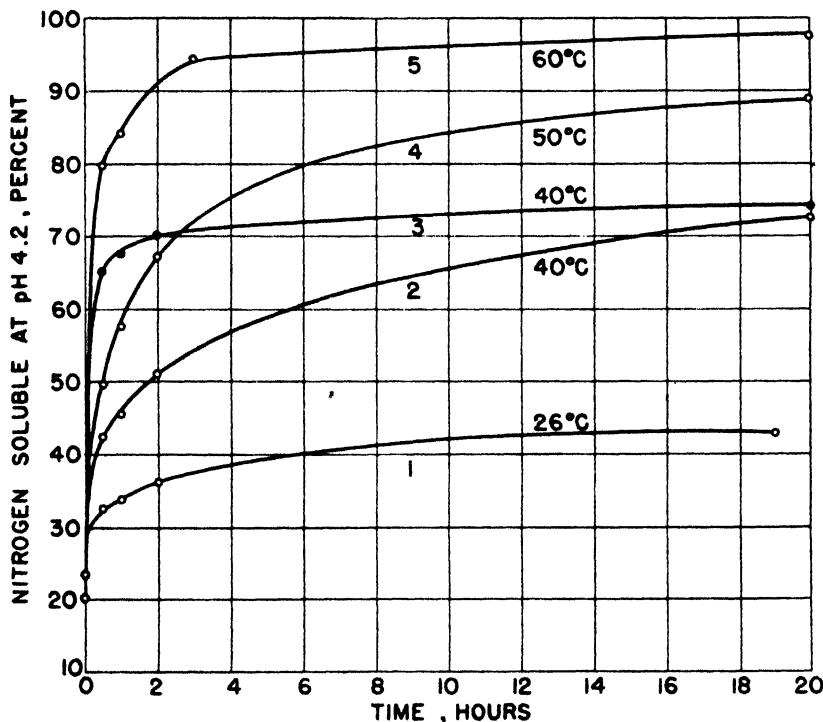


Fig. 5. The effect of temperature on rate of reaction at E:S nitrogen ratio of 1:15 and at pH 6.6. Curve 3, the protein solution, was boiled 5 minutes prior to the hydrolysis. The higher of the two zero values is for the curves at 50° and 60°C.

In Fig. 3, curves 2 and 4 represent reactions at 26° and 40°C., respectively, without deactivators; in curve 1 the hydrogen peroxide was added to the protein before the enzyme and in curve 3, 15 minutes after the enzyme.

Effect of Temperature. The data on effect of temperature on papain activity are shown in Fig. 4 for E:S nitrogen ratio of 1:60 and in Fig. 5 for E:S nitrogen ratio of 1:15. In these experiments no activator was used and the crude enzyme was added to the protein dis-

persion after it had been adjusted to the indicated temperature. At the time intervals shown in Fig. 5 a sample was withdrawn from the system, the enzyme deactivated with hydrogen peroxide, and the nitrogen precipitated at pH 4.2. The results show a very rapid increase in proteolysis with increase in temperature. In Fig. 5, for example, the raising of the temperature from 26°C. to 60°C., and for a 2-hour reaction time, the increase in soluble nitrogen amounts to 55%. Curve 3, Fig. 5, shows the reaction on a protein sample previously boiled for 5 minutes as compared to an unboiled sample shown in curve 2. The difference between these two reactions is largely in the early stages.

The enzyme activity is slowly destroyed at 80°C. In another experiment not recorded here, it was found that at 95°C. it required between 15 and 30 minutes to destroy the enzyme completely. However, in considering the rate of destruction of an enzyme with heat, the results may be somewhat different for an isolated enzyme than for an enzyme substrate mixture.

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STUDIES ON BREAD STALING. III. INFLUENCE OF FERMENTATION VARIABLES ON BREAD STALING AS MEASURED BY COMPRESSIBILITY AND FARINOGRAPH PROCEDURES¹

J. FREILICH²

ABSTRACT

The crumb compressibility and the farinograph consistency procedures were used simultaneously in studying the effects of several fermentation variables and related factors on the staling of bread. The procedures are described and their relative accuracy compared.

The factors studied were variations in amount of yeast at constant dough volume and at constant fermentation time, variations in fermentation time of straight doughs and sponge doughs, and the effects of a crumb-softening agent.

No really significant effects on the rate of staling were produced by the fermentation variables so far studied, under the conditions used in this work.

Compressibility and farinograph data showed satisfactory agreement on the effects of fermentation variables on the rate of staling. There was some disagreement on apparent freshness. Where large differences in volume were involved, only the compressibility values indicated comparable differences in softness, which is said to be one criterion of freshness or consumer preference. There was definite disagreement on the effects of a crumb-softening agent; the compressibility procedure showed that this agent produced a definite decrease in the rate of staling, while the farinograph procedure showed a slight tendency in the opposite direction.

It is suggested that both procedures are of value in the study of bread staling, and that they be used simultaneously in continued studies on this problem.

The Committee on Food Research, of the Quartermaster Food and Container Institute for the Armed Forces, has been investigating the general problem of staling in bread. As a part of this work, the Fleischmann Laboratories have been conducting a study on the effects of type and extent of fermentation on the rate of staling.

Since there is no generally accepted method for measuring bread staling, it appeared advisable to compare available procedures for this purpose, among which are the crumb compressibility and the farinograph consistency procedures.

Several questions came to mind in this connection. To what extent do these procedures agree? If they don't agree, which is preferable? Which is more accurate? Which gives better correlation with consumer preference?

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² The Fleischmann Laboratories, Standard Brands Inc., New York.

Preliminary work had indicated some disagreement between the compressibility and farinograph consistency procedures. It was therefore decided to use both procedures simultaneously in studying the effects of some fermentation variables on the rate of staling.

Materials and Methods

Baking Procedure. The following formula was used:

Flour	100% (1800 g.)
Water	64 to 66%
Yeast (variable)	2%
Sugar	5%
Diamalt	1%
Salt	2%
Shortening	3%
Milk powder	3%
Arkady	0.25%

Six loaf sponge or dough batches were mixed in the Readco mixer. Amounts of ingredients were based upon standard size (1 pound) loaves.

The sponges (60% of the flour, 37% water, the yeast, and the Arkady) were fermented at 30°C. for 3½ hours, then mixed with the balance of the dough ingredients at 30°C.; after 30 minutes the dough was divided into six equal portions, which were rounded, molded by machine after a 15-minute rest period, proofed to the top of the pan at 40°C., and baked at 215°C. for 30 minutes.

The straight doughs were fermented for about 2 hours (to a dough volume of 1180 ml. for a one-loaf dough), divided into six equal portions, rounded, molded 15 minutes later, then proofed and baked as in the sponge-dough procedure.

Two of the loaves were used for farinograph consistency measurements, and the remaining four loaves for compressibility measurements.

Compressibility Method. Changes in compressibility of bread are used as a measure of the rate of staling, since stale bread is much less compressible than fresh bread. Compressibility was measured by noting the extent of penetration of a plunger (1.9 cm. in diameter, with a spherical end) into a cube of bread crumb under a fixed stress and standard conditions, as described below. The type of apparatus used is a modified rough balance, described by Platt (3). A cube of bread measuring about 1½ inches thick and 2½ inches square is placed on a flat surface; the counterbalanced plunger is brought down so it just touches the surface of the bread, and a movable scale marked in millimeters is placed behind the balance pointer at the zero reading. Stress is exerted on the plunger by slowly admitting 100 ml. of water through a small opening into a container resting on top of the plunger,

which has been counterbalanced with it. The water flows into the container in 20 seconds, and extent of penetration is measured 3 minutes later. The first reading is made on the fresh bread 1½ hours after removal from the oven; readings thereafter are made at about 24, 48, and 72 hours.

Results representing averages of readings on four replicate loaves are expressed as compressibility, in millimeters, and as change in compressibility, in per cent; the first reading is taken as 100% compressibility. During the test, the bread is kept in double wax paper to avoid excessive loss of moisture. The bread is stored at room temperature.

Farinograph Method. The farinograph procedure used is based on one originally suggested by Fuller (1), and recently developed by Geddes and collaborators (2). The method makes use of the fact that the consistency of bread crumb and water "doughs," at constant moisture, decreases as bread undergoes staling changes.

A small farinograph bowl (50 g. capacity) was used to measure changes in maximum farinograph consistency of bread crumb and water "doughs" having a total weight of 80 g. and a moisture content of 60%. A portion of the loaf to be measured was cut off, and the rest of the loaf wrapped in double wax paper and saved for future measurements. The crumb was removed from the cut portion, and shredded in the Waring Blender; the moisture of the shredded crumb was determined, and farinograph curves made with amounts of crumb and water calculated to make a "dough" of 60% moisture. Farinograph curves were made when the bread was one hour out of the oven, and about 24, 48, and 72 hours thereafter. Since the moisture determination required about 2 hours, the exact moisture content was not known at the time the fresh (one-hour) bread was tested; approximate amounts of crumb and water, at two levels in the region of 60% moisture, were therefore used, and a farinograph consistency value calculated for 60% from the two observed values, after the moisture of the crumb was determined.

Tests were made in duplicate, and results recorded as farinograph consistency, and per cent change in farinograph consistency, with the original value on the one-hour bread taken as 100%.

Results

Accuracy of Compressibility and Farinograph Procedures. The relative accuracy of the two procedures is indicated in Table I, which shows the mean values and standard errors obtained by both procedures on loaves from the same dough batch. In these experiments, doughs with 0.5, 1, and 2% yeast were subjected to constant fermentation

TABLE I

COMPRESSIBILITY AND FARINOGRAPH CONSISTENCY DATA FOR STRAIGHT DOUGH BREAD WITH DIFFERENT AMOUNTS OF YEAST AND CONSTANT FERMENTATION TIME

Amt. of yeast (%)	Age of bread (days)											
	0			1			2			3		
	COMPRESSIBILITY (MM.)											
Amt. of yeast (%)	Mean	S	Per cent error	Mean	S	Per cent error	Mean	S	Per cent error	Mean	S	Per cent error
0.5	9.1	0.46	5.1	4.6	0.224	4.9	4.3	0.283	6.6	3.2	0.152	4.7
1.0	14.1	1.88	13.3	7.4	0.69	9.3	5.2	0.10	1.9	5.0	0.14	2.8
2.0	21.8	1.17	5.4	10.4	0.35	3.4	8.3	0.19	2.3	7.5	0.27	3.6

FARINOGRAPH CONSISTENCY (B.U.) AT 60% MOISTURE												
0.5	— ¹	230	0	0	212.5	3.5	1.7	195	0	0		
1.0		245	7.07	2.9	235	7.07	3.0	205	0	0		
2.0		275	7.07	2.6	245	7.07	2.9	225	7.07	3.1		

¹ The farinograph readings on the fresh bread (0 days) were not averaged, because they were obtained at different moisture levels, in order to serve as a basis for calculating a value for 60% moisture (see farinograph method in text).

NOTE: S = Standard error (or standard deviation, as defined in *Cereal Laboratory Methods*, 5th Ed., 1947). Per cent error = $\frac{S}{\text{mean}}$. B.U. = Brabender Units. Each compressibility mean represents four individual readings and each farinograph mean represents two individual readings.

time. Each compressibility value represents four individual readings, and each farinograph value represents two individual readings.

The compressibility readings on the fresh bread (0 days) usually show the greatest errors, or deviations from the mean values. The standard errors for 0.5, 1, and 2% yeast at 0 days (Table I) were, respectively, 0.46, 1.88, and 1.17 mm., or 5%, 13%, and 5% of the mean values. Thirteen per cent is, of course, a large error; in this set of readings the maximum error was much greater than the standard error; if the one reading which showed this large error had been discarded, the standard error would have been greatly reduced. Errors of a similar order of magnitude are not infrequent in compressibility measurements, however, and it is therefore important to keep this in mind in interpreting compressibility data.

The compressibility values for 1% yeast at 1 day showed a standard error of 9%; this was the largest deviation found outside of the readings for the fresh bread (0 days). Comparable deviations for the farinograph consistency values were very much smaller (Table I).

The farinograph readings for fresh bread (Table I) could not be averaged because they were obtained at different moisture levels. An experiment was therefore conducted in which six replicate loaves were used in farinograph measurements at constant moisture (60%) when the bread was 3 to 3½ hours old, and the crumb moisture had been determined. For comparative purposes, portions of the same loaves were also used in compressibility measurements. The results are shown in Table II.

TABLE II

INDIVIDUAL COMPRESSIBILITY AND FARINOGRAPH CONSISTENCY READINGS ON SIX REPLICATE LOAVES OF FRESH BREAD MADE FROM STRAIGHT DOUGH¹

Loaf no.	Compressibility (mm.)	Farinograph consistency ² (B.U.)
1	17.5	440
2	18.5	420
3	18.1	420
4	18.3	420
5	18.7	435
6	18.5	425
Mean	18.3	427
Standard error (S)	0.43	8.75
Per cent error $\left(\frac{S}{\text{mean}}\right) \times 100$	2.35	2.05

¹ Age of bread, 3 to 3½ hours.

² Farinograph consistency at 60% moisture.

The standard error for the farinograph values was 8.75 B. U. or 2.05%. Surprisingly, the standard error for the compressibility values was 0.43 mm. or 2.35%, an unusually accurate result, considering the errors generally encountered in compressibility data.

From all the above data, it seems apparent that the farinograph procedure is inherently more accurate than the compressibility procedure. It has been observed, however, that the accuracy of the compressibility procedure is susceptible of improvement with experience. If the technician is careful to avoid placing the plunger immediately above large holes or hard lumps on the crumb surface, the errors involved may be distinctly minimized. The cube of bread crumb should be cut after the measurement to observe the possible presence of large holes under the surface which were not visible when the test was made.

Effects of Varying Amounts of Yeast in Doughs Fermented to Constant Volume. The results of tests with 0.5, 1, and 2% yeast in straight doughs fermented to constant volume are shown in Fig. 1. The findings by both procedures may be outlined as follows:

Compressibility

2% yeast produced the highest values, or best apparent freshness.

The effects of yeast on compressibility were irregular.

The amount of yeast used did not change the rate of staling (per cent change in compressibility).

Farinograph Consistency

2% yeast produced the highest farinograph values, or best apparent freshness.

The effects of yeast on farinograph consistency were progressive (with amount of yeast).

The amount of yeast used did not change the rate of staling.

The two procedures here showed satisfactory agreement on two important points: (1) apparent freshness and (2) rate of staling. They did not agree in that compressibility values showed an irregular trend, whereas the farinograph values showed a progressive trend with increasing amounts of yeast; this seems to be a point in favor of the farinograph procedure.

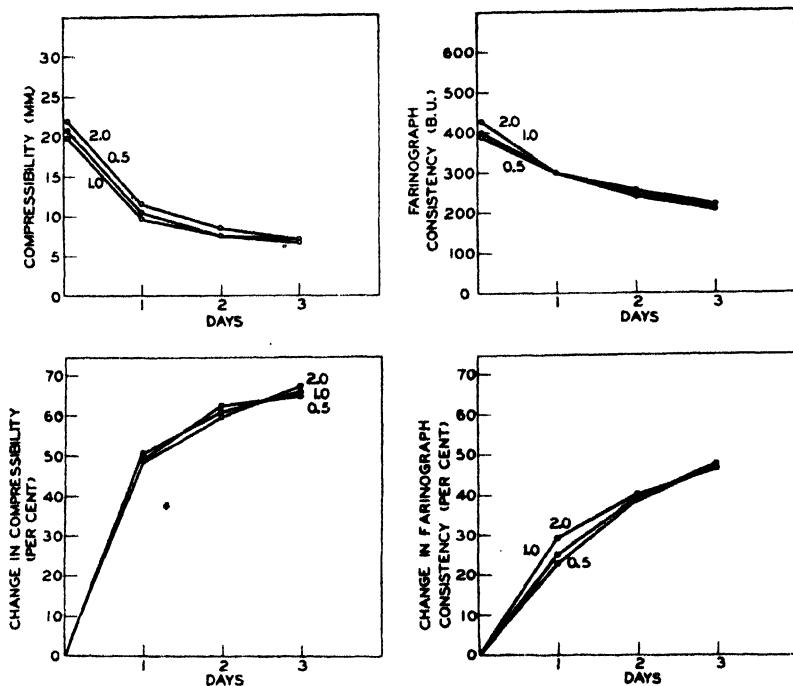


Fig. 1. Effects of 0.5, 1.0, and 2.0% yeast on compressibility and farinograph consistency of bread made from straight doughs fermented to constant volume.

Varying Amounts of Yeast in Doughs with the Same Fermentation Time. Fig. 2 shows the results of straight dough tests with 0.5, 1, and 2% yeast, all fermented for the same time, that which is normally

used for 2% yeast. The doughs with 0.5 and 1% yeast were, of course, greatly underfermented and underproofed; this was reflected in correspondingly poorer loaf volume, texture, and grain.

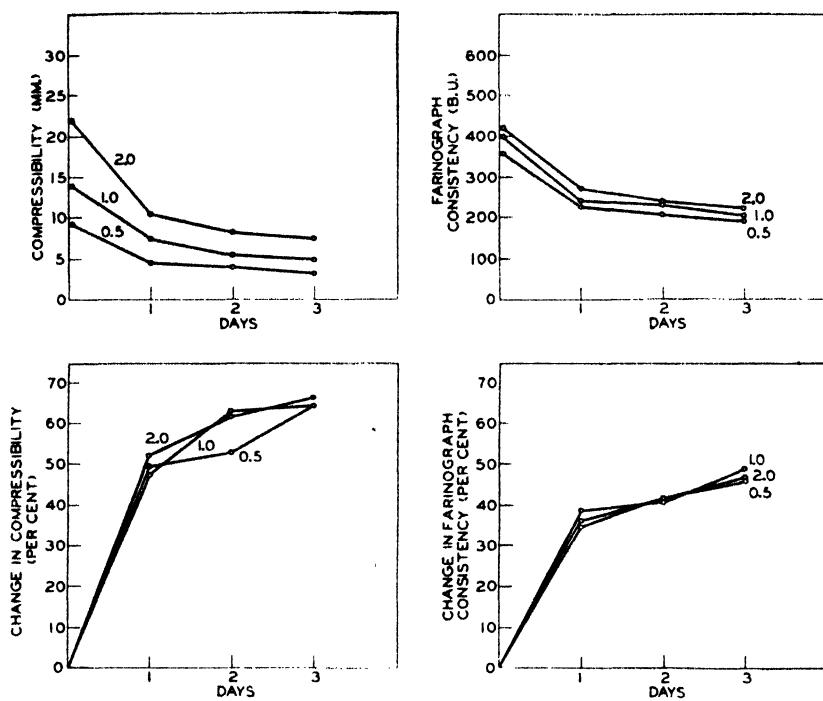


Fig. 2. Effects of 0.5, 1.0, and 2.0% yeast on compressibility and farinograph consistency of bread made from straight doughs fermented for the same time.

The relative findings were as follows:

Compressibility

The 2% bread was by far the most compressible, throughout the test period.

Compressibility varied as the amounts of yeast used.

The differences due to the amount of yeast used were very great.

Staling rate differences were insignificant.

There was good agreement here, except for one rather important point, the degree of difference produced. The compressibility pro-

Farinograph Consistency

The 2% bread showed greater farinograph consistency values throughout the test period.

Farinograph consistency varied as the amounts of yeast used.

The differences due to the amount of yeast used were slight by comparison with those shown by the compressibility values.

Staling rate differences were insignificant.

cedure showed great differences, in line with the variations in amount of yeast used, and with the resulting differences in volume and texture; but the differences in farinograph consistency were relatively slight. Since compressibility is a direct measure of softness in bread, which in turn is an important criterion of consumer preference, it is obvious that the compressibility procedure is preferable as an indicator of apparent freshness.

However, the compressibility values in this experiment serve as a good illustration of how misleading softness may be as an index of freshness. We know that the 0.5% bread was just about as fresh as the 2% bread, since both were baked on the same day (about 1½ hours apart), and yet, on the basis of compressibility values or softness alone, the 2% bread would be considered much fresher than the 0.5% bread.

Effects of Variations in Fermentation Time of Straight Doughs. Straight doughs with 2% yeast were fermented for ½, 2, and 4 hours, respectively, then proofed to normal volume (the top of the pan) and baked. The results are shown in Fig. 3.

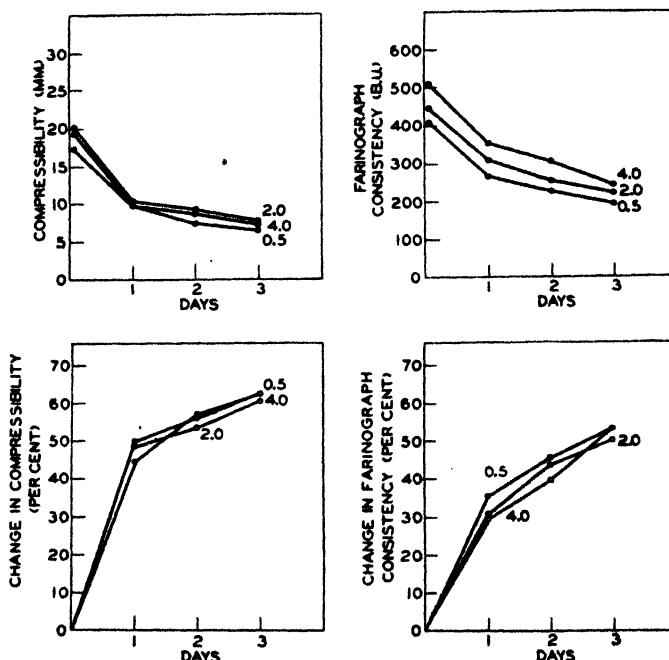


Fig. 3. Effects of 0.5, 2.0, and 4.0 hour dough times on compressibility and farinograph consistency of bread made from straight doughs.

The relative findings may be listed as follows:

Compressibility

The 2-hour fermentation produced the most compressible bread.

The differences due to fermentation time changes were irregular.

The 4-hour fermentation produced greater compressibility than the 3-hour fermentation, although the latter gave greater loaf volume.

There was no significant effect on the rate of staling.

Farinograph Consistency

The 4-hour fermentation produced the highest farinograph consistency.

The differences due to fermentation time changes were progressive.

The differences were relatively greater than those shown by the compressibility values.

The 4-hour fermentation produced a slight reduction in staling for the first 2 days, but there was no difference in rate at the end of 3 days.

The agreement between compressibility and farinograph consistency for these tests was not good. The compressibility values in-

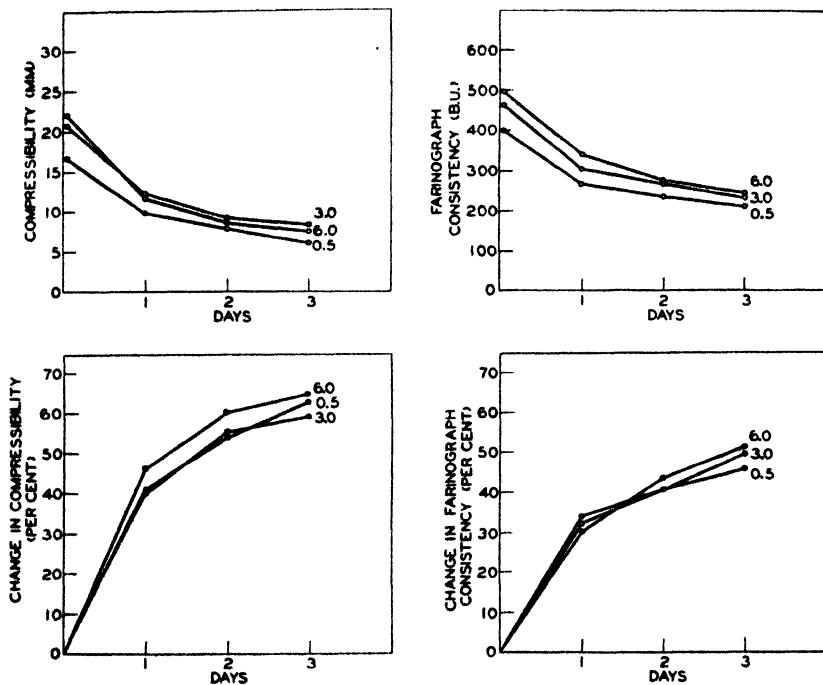


Fig. 4. Effects of 0.5, 3.0, and 6.0 hour sponge fermentation times on compressibility and farinograph consistency of the bread produced.

dicated the best apparent freshness for the 2-hour fermentation, which also produced the best loaf volume and bread quality, and would thus be preferable from the consumer viewpoint. On the other hand, the farinograph values indicated that the 4-hour fermentation produced

the best apparent freshness, and showed better correlation with fermentation changes throughout.

Effects of Variations in Sponge Fermentation Time. Sponges with 2% yeast were fermented for $\frac{1}{2}$, 3, and 6 hours, respectively, then re-mixed, proofed, and baked as indicated in the procedure. The results are shown in Fig. 4.

The relative findings were as follows:

Compressibility

The 3 and 6 hour breads were the most compressible, with the 3 hour bread the better of the 2 except at 0 days.

The trend in compressibility values was irregular as regards sponge time, but varied as loaf volume.

The 6-hour fermentation produced a slight increase in the rate of staling during the first 2 days.

Farinograph Consistency

The 6 and 3 hour breads produced the highest farinograph consistency values, with a slight advantage in favor of 6 hours throughout the test period.

The trend in farinograph values was progressive with respect to sponge time.

The 6-hour fermentation increased the rate of staling to a slight extent during the 2nd and 3rd days, as compared to the $\frac{1}{2}$ -hour fermentation.

The agreement between the two procedures was fair in these tests; both showed the beneficial effects of sponge fermentation on apparent freshness, and both showed a tendency for the 6-hour fermentation to increase staling to a slight though unimportant extent.

Effects of a Crumb-Softening Agent. A product recently marketed as a crumb softener³ for yeast-raised goods was tested in sponge-doughs at levels of 0.5 and 1%. The results are given in Fig. 5.

The following were the relative findings by the two procedures:

Compressibility

The softener increased compressibility.

The trend was progressive (except for the fresh bread).

The differences in compressibility were fairly large.

There was a significant decrease in rate of staling.

Farinograph Consistency

The softener decreased farinograph consistency.

The trend was progressive.

The differences in farinograph consistency were distinct.

There was a slight though perhaps insignificant increase in the rate of staling.

These results showed a definite disagreement between the two procedures. There was a negative correlation between the effects of the softener, one procedure showing an increase, and the other a decrease in apparent freshness. The most important difference, however, was shown by the per cent change in the values obtained by both procedures. The compressibility values indicated a significant inhibition

³ A polyoxyethylene stearate with an average chain length of nine oxyethylene residues per molecule.

in the rate of staling due to the softening agent, whereas an opposite though less significant trend was indicated by the farinograph values. Though both procedures show evidence of change, only the change in compressibility or softness would be discerned by the consumer. On the basis of consumer preference, therefore (assuming that softness is a valid criterion of preference), the compressibility procedure appears to have an important advantage over the farinograph procedure. However, the very fact that the farinograph data are in complete dis-

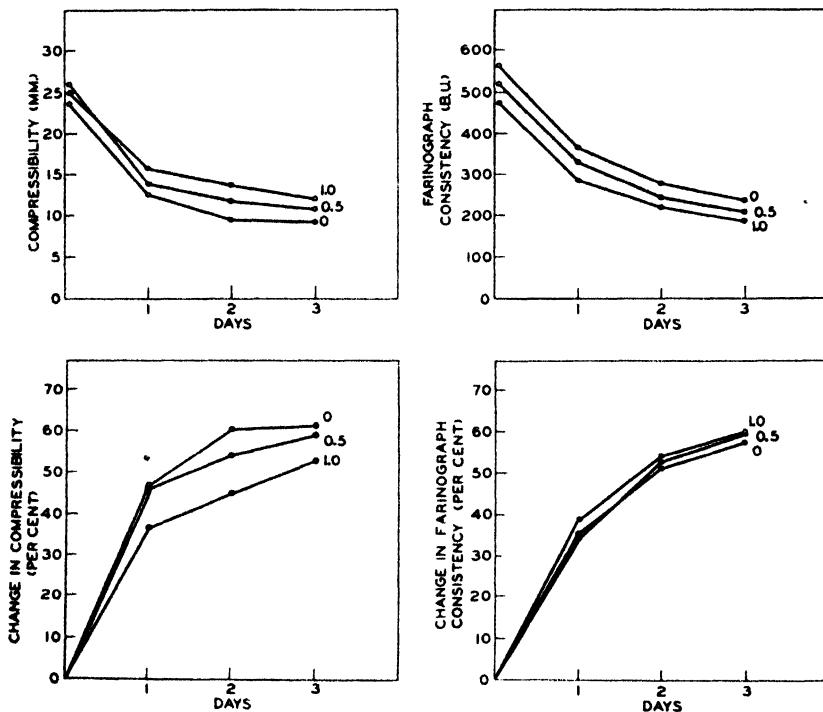


Fig. 5. Effects of 0, 0.5, and 1.0% of a softening agent on compressibility and farinograph consistency of sponge-dough bread.

agreement on this point is sufficient to cast some doubt on the true nature of the change indicated by the compressibility values.

Discussion

Compressibility and farinograph data were essentially in agreement on apparent freshness and rate of staling as influenced by several fermentation variables.

The following points of disagreement were noted: (1) In testing the effects of $\frac{1}{2}$, 2, and 4-hour dough times, the best apparent freshness was obtained with the 2-hour dough time, according to com-

pressibility data, and with the 4-hour dough time by farinograph data. (2) The variables studied always produced progressive trends in the farinograph values, but the trends in compressibility values were irregular, and apparently were influenced by volume and texture. (3) The extent or degree of difference was much greater in the compressibility values when the volume differences were large. Where doughs with different amounts of yeast were fermented for the same time, the larger loaves were much more compressible or softer; the farinograph differences for similar loaves were relatively slight. (4) There was definite disagreement between the two procedures on the effects of a softening agent. Here the compressibility values showed increased softness and a definitely lowered rate of staling, but the farinograph values showed decreased consistency and a slightly higher staling rate due to the softening agent. The validity of crumb softness alone as a criterion of freshness is, of course, open to question. Variations in volume or type of bread may produce differences in softness when there is no difference in freshness. Flavor is also a factor in freshness. It is quite conceivable that a heavy, compact loaf may retain volatile flavors longer because less surface for evaporation is exposed than in a larger but otherwise similar loaf.

It is indicated that there was not enough agreement between the procedures to justify the use of either one alone. There was, however, sufficient agreement to show that both procedures were of value. The compressibility procedure showed better correlation with softness, which is one aspect of consumer preference. The compressibility procedure is also less time consuming than the farinograph procedure. The farinograph procedure is more accurate, and produced more uniform trends with respect to the effects of the variables studied.

Perhaps the most important consideration is that both procedures measure changes which are progressive in time, and of a roughly similar order of magnitude; these changes are probably related to true staling. If a factor is found which inhibits staling significantly by both procedures, one might be fairly certain that true rather than apparent freshness is involved.

Acknowledgment

The author wishes to express his thanks to R. F. Light, Manager, and G. W. Kirby, Technical Director, of the Fleischmann Laboratories, for their helpful interest, and to Miss Joan Bachman and Harold Wagner for their valued assistance in this work.

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CHEMICAL STUDY OF THE MATURE WHEAT KERNEL BY MEANS OF THE MICROSCOPE¹

M. R. SHETLAR²

ABSTRACT

The composition of the wheat kernel was investigated by microchemical methods *in situ*. Cellulose is present in large amounts in the walls of the epidermal, cross layer, and hyaline cells. Some is present in the walls of the aleurone cells, but only a small amount occurs in the testa layer. Lignin occurs in the middle lamella of the cross layer cells, in some of the epidermal cell walls, and in the wheat hairs. A layer of cutin is present on the epidermis of the wheat kernel, and the testa cell walls are heavily cutinized. Lipid material is present within the aleurone cells. Pectic compounds appeared to be present in all of the bran layers, being located either in the middle lamellae or in the cell walls adjacent to them. Protein material was found largely in the testa, aleurone cell contents, and starchy endosperm, being especially high in the aleurone layer.

As the structure of the wheat kernel is of considerable practical importance to the agronomist and to the milling technologist, the microscopic appearance of the wheat kernel has been extensively studied and reviewed (3, 4, 5, 6, 11). However, aside from several studies of the development of the wheat kernel (1, 2, 7, 9), microchemical methods were not extensively employed. It is the purpose of this paper to present the results of a preliminary microchemical study *in situ* of the mature wheat kernel.

Materials and Methods

A sample of Ohio soft red winter wheat and a sample of Michigan white wheat were used to furnish kernels for the work. Cross and longitudinal sections were made by soaking the grain for about 4 hours in water and then sectioning it with the freezing microtome. Sections of approximately 20 μ in thickness proved to be most satisfactory. Flat sections of the various bran layers were obtained either by stripping them by hand from the kernel or by chemical separation as previously described (10).

Photomicrographs were originally made in natural color using a Leitz photomicrographic camera with a special adapter for 35 mm. film, and a Bausch and Lomb polarizing microscope. Photographs presented here are black and white transfers made from the original kodachrome transparencies.

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Microchemical tests used were those compiled by Sampson (8) and may be divided into four types: (1) tests depending upon differential staining, (2) tests depending upon specific color reactions, (3) tests based upon solubilities, and (4) tests based upon optical properties. The tests may also be classified as follows according to the constituent which is being studied.

Cellulose. A blue or violet color reaction occurs when tissue containing cellulose is placed in the chloro-zinc-iodide reagent (25 g. zinc chloride, 8 g. potassium iodide, 1.5 g. iodine, 8 ml. water). Cellulose lamellae are soluble in ammoniacal cupric hydroxide (ammonium hydroxide is added to saturated copper sulfate solution until solution appears green, then filtered; the precipitate is washed with water, dried, and dissolved in ammonium hydroxide solution). Cellulose lamellae are also soluble in 75% sulfuric acid solution. Cellulose lamellae are anisotropic under crossed nicols, usually with positive elongation when observed in cross and longitudinal sections of tissue.

Cutin and Suberin. Tissue containing either cutin or suberin are stained by Sudan IV (0.1 g. Sudan IV, 5 ml. 95% ethanol, and 5 ml. glycerol) when placed in the dye solution for some time and then washed with 50% ethanol. If sulfuric acid is added to tissue thus stained, the stained areas become blue. Cutin and suberin are soluble in a 10% solution of potassium hydroxide in either ethanol or glycerol after heating for one hour. Droplets of ceric acid appear in sections containing cutin or suberin upon treatment with Schultz's reagent (equal volumes of nitric acid and potassium chlorate solution) followed by gentle heating. Suberin is distinguished from cutin by the potassium phellonate test which consists of macerating the tissue with saturated potassium hydroxide solution for several hours, heating mildly, and finally heating to a boil. The potassium phellonate appears as granular masses which give a violet-red color reaction with the chloro-zinc-iodide reagent. Suberin lamellae are anisotropic, while pure cutin lamellae are isotropic; however cutinized cellulose lamellae are anisotropic with negative elongation.

Pectic Compounds. Pectic compounds may be differentially stained by placing a section in ruthenium red solution (one part ruthenium red to 10,000 parts of aqueous weakly ammoniacal solution) for about 20 minutes, followed by thorough washing with water. All pectic compounds are soluble when heated with dilute acids (2% hydrochloric acid solution) for 30 to 60 minutes and then treated with 2% potassium hydroxide solution. Pectic lamellae, cutinized pectic lamellae, and lignified pectic lamellae are isotropic under crossed nicols.

Pentosans. Tissues containing pentosans undergo a red to violet color reaction when a section is placed in a solution of phloroglucin

(1% in ethanol) for a few minutes and then is placed in a drop of concentrated hydrochloric acid and heated gently for 10 minutes.

Lignin. A red color reaction is obtained without heating with the phloroglucin-hydrochloric acid test as described for pentosans when lignin is present. Lignified pectic lamellae are isotropic under crossed nicols, while lignified cellulose lamellae are anisotropic with positive elongation.

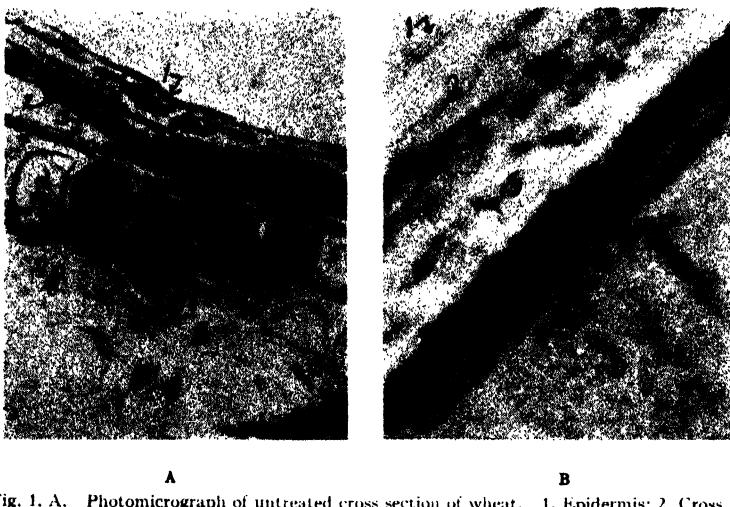


Fig. 1. A. Photomicrograph of untreated cross section of wheat. 1. Epidermis; 2. Cross Layer; 3. Testa; 4. Hyaline; 5. Aleurone; 6. Starchy Endosperm. $\times 200$.
 B. Longitudinal section of wheat under polarizing microscope using selenite red plate. Black and white transfer was made using blue filter. Under these conditions outer part of epidermis (1) which is isotropic appears dark. Rest of epidermis which is blue in kodachrome print appears as more or less light area (2). Cross layers (3) likewise appear as light area. Testa (4) also being isotropic appears dark. Hyaline layer (5) which is blue in kodachrome print appears beneath testa layer. Part of an aleurone cell (6) is visible. $\times 900$.

Proteins. The xanthoproteic test was used by placing the section in a drop of nitric acid solution (3-1 in water), drawing off the excess acid and adding a drop of ammonium hydroxide solution. Tissue containing protein becomes yellow to brown in color. Proteinaceous material in sections placed in a saturated aqueous solution of picric acid for some time gives a typical yellow reaction. The biuret test for protein may be used by placing sections in 5% cupric sulfate solution for 30 minutes, washing, and adding saturated potassium hydroxide solution. The protein material is stained a red to blue color.

Results

In all of the following, the various layers of the bran and the adjacent endosperm are labeled according to the nomenclature given in Fig. 1A.

Optical Properties. In cross or longitudinal sections of the wheat kernel, the outer layer of material of the epidermis was isotropic, being invisible under crossed nicols or red if the selenite red plate was used (Fig. 1B). The rest of the cell walls of the epidermis were anisotropic and appeared blue when placed parallel to the plane of vibration of the selenite red plate in both cross and longitudinal sections. When viewed in a flat section, the majority of the cell walls at the butt and brush ends of the epidermis had a negative elongation, appearing yellow when parallel to the plane of vibration of the selenite red plate. On the other hand, most of the cell walls of the center portion of the epi-

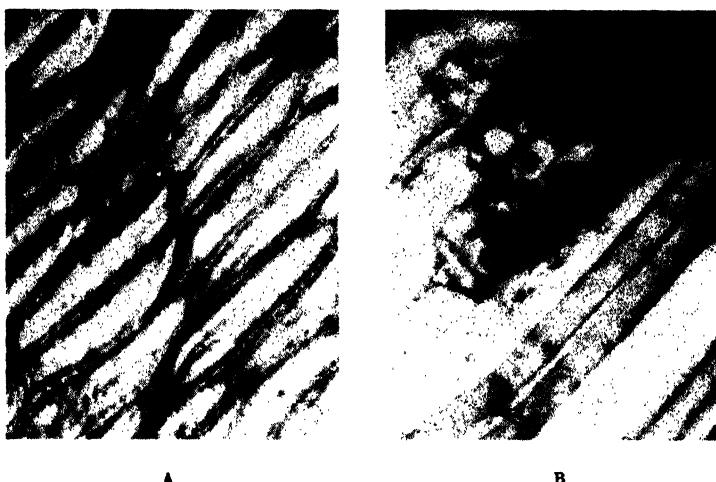


Fig. 2. A. Photomicrograph of flat section of epidermis under crossed nicols using selenite red plate. Long axes of cells are parallel to the plane of vibration of selenite red plate. Dark portions are blue. $\times 200$.

B. Section of wheat hair under crossed nicols parallel to the section of vibration of selenite red plate. Dark portions are blue. $\times 900$.

dermis had a positive elongation. After treatment with the glycerol-potassium hydroxide reagent the middle lamella of the epidermis cells was swollen and appeared isotropic, and most of the remaining cell walls exhibited positive elongation (Fig. 2A). When placed parallel to the plane of vibration of the selenite red plate, the cell walls of the wheat hairs appeared blue under crossed nicols (Fig. 2B).

The walls of the cross layer cells were anisotropic under crossed nicols. When viewed parallel to the plane of vibration of the selenite red plate, they appeared yellow in a cross section of the wheat kernel, while in the longitudinal section they appeared blue under the same conditions. In a flat strip section (Fig. 3A) the inner walls of the cross layer cells appeared yellow, while the outer walls and middle lamella appeared purple when viewed parallel to the plane of vibration of the

selenite red plate. After treatment of these cells with potassium hydroxide in glycerol, all cell walls exhibited negative elongation. Treatment with ammoniacal cupric hydroxide 'solution' destroyed the

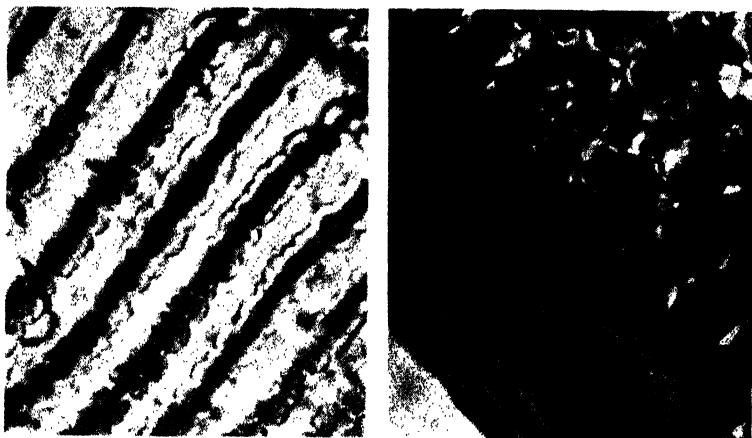


Fig. 3. A. Photomicrograph of flat section of cross layer cells under crossed nicols. Long axes of cells are parallel to the plane of vibration of selenite red plate. Dark areas are purple; light areas are yellow. $\times 900$.
B. Flat section of aleurone and hyaline cells under crossed nicols. Hyaline cells are the elongated cells to the left and have their long axes parallel to selenite red plate. Filled aleurone cells are in lower right hand corner; empty aleurone cells appear above them. Except for the aleurone cell contents which are naturally dark, the dark areas are blue and the light areas are yellow.

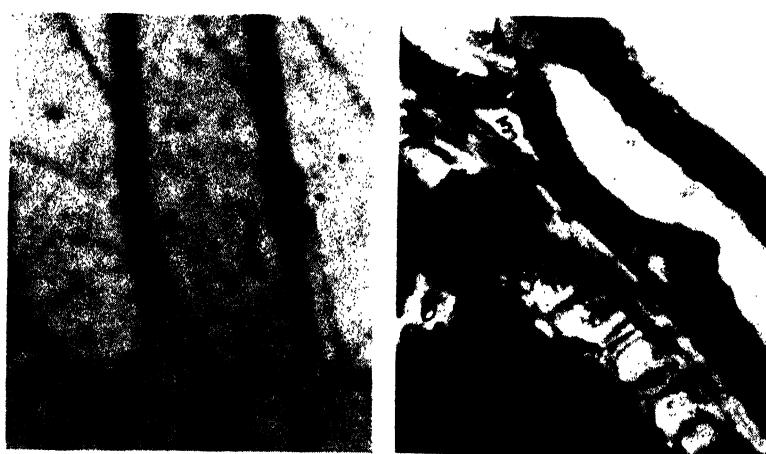


Fig. 4. A. Photomicrograph of flat section of testa layers of white wheat after treatment with chloro-zinc-iodide reagent. Cell walls react faintly and appear dark in photograph. Outer layer of testa (in focus) is superimposed on inner layer (out of focus). $\times 900$.
B. Photomicrograph of cross section of wheat after treatment with chloro-zinc-iodide reagent. Tissue containing cellulose becomes blue and appears dark in photograph. Starch also reacts so that starch endosperm (6) also appears dark. Being brown, testa (3) appears dark although it did not react with reagent. $\times 200$.

anisotropy of the cross layer cells. The cell walls of the tube cells had optical properties similar to those of the cross layer cells.

The walls of the testa cells were unique in that they were isotropic under crossed nicols.

The hyaline and aleurone cell walls were anisotropic (Fig. 3B). The aleurone cell contents were isotropic under crossed nicols. When viewed parallel to the plane of vibration of the selenite red plate, the cell walls of the hyaline cells appeared blue while the middle lamella

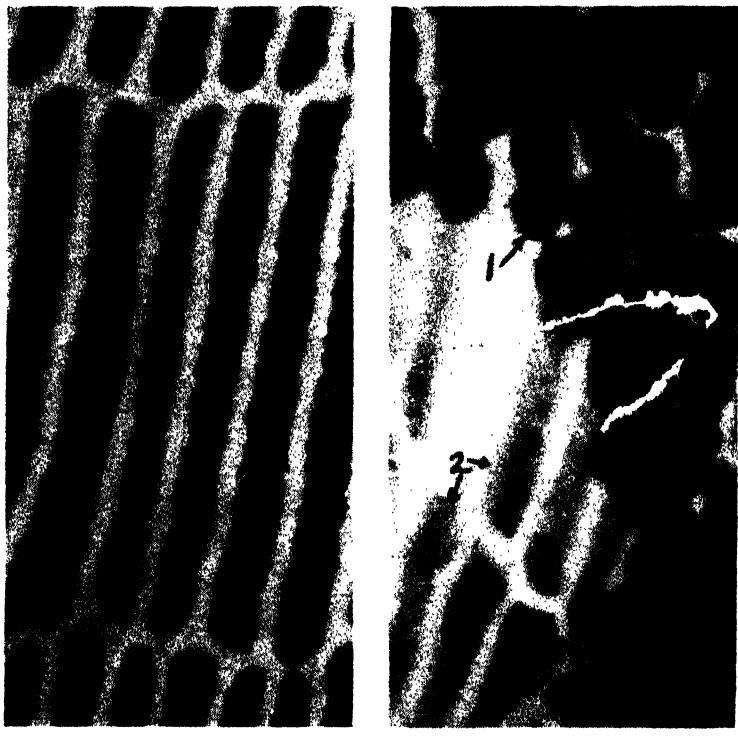


Fig. 5. A. Photomicrograph of flat section of cross layer cells of wheat after treatment with chloro-zinc-iodide reagent. Tissues containing cellulose become blue and are dark in photograph. $\times 900$.
B. Photomicrograph of flat section of aleurone cells (1) superimposed upon hyaline cells (2) after treatment with chloro-zinc-iodide reagent. $\times 200$.

was yellow. Aleurone cell walls parallel to the selenite red plate also appeared blue.

The cell walls of the starchy endosperm were anisotropic and were blue when viewed parallel to the plane of vibration of the selenite red plate.

Cellulose. With the possible exception of the testa layer, cellulose occurs in generous amounts in the various bran layers, as shown by the

chloro-zinc-iodide test carried out either on the original preparation or after treatment with potassium hydroxide in glycerol. The testa of red wheat either failed to react with the reagent or the reaction is



Fig. 6. A. Photomicrograph of wheat epidermis flat section after treatment with glycerol-potassium hydroxide solution and then with chloro-zinc-iodide reagent. $\times 200$.
 B. Wheat hairs after treatment with chloro-zinc-iodide reagent. $\times 200$.



Fig. 7. A. Photomicrograph of longitudinal section of wheat kernel after treatment with ruthenium red solution. Transfer was made with blue filter so that pink areas appear dark. $\times 200$.
 B. Flat section of white wheat testa after treatment with ruthenium red solution. Pink areas appear dark.

masked by the brown pigment of this layer. Strip sections of white wheat testa, however, reacted mildly with chloro-zinc-iodide (Fig. 4A). When a cross section of the wheat kernel was treated with the reagent,

the walls of the epidermis, the cross layer cells, the hyaline, and the aleurone cells gave a strong reaction (Fig. 4B). In strip sections the walls of the cross layer (Fig. 5A), the hyaline (Fig. 5B), and the aleurone (Fig. 5B) cells all reacted with chloro-zinc-iodide without

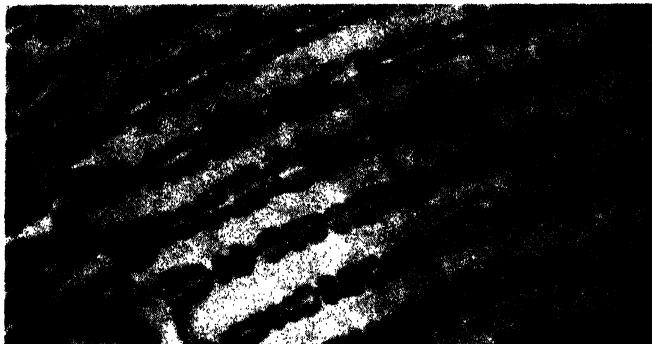


Fig. 8. Photomicrograph of flat section of cross layer cells after treatment with ruthenium red solution. Pink areas appear dark. $\times 900$.

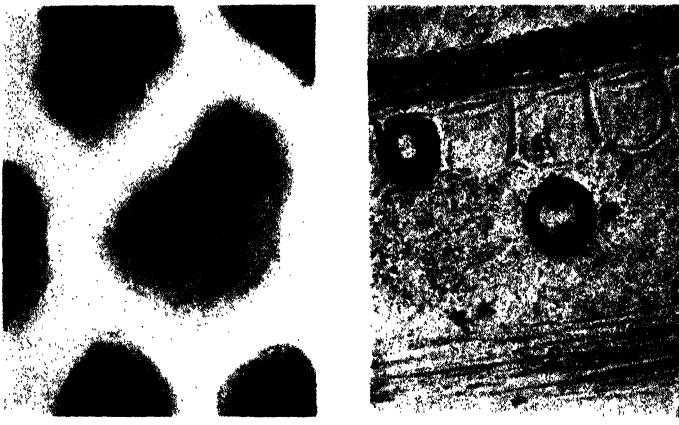


Fig. 9. A. Photomicrograph of flat section of aleurone cells after treatment with ruthenium red solution. $\times 900$.

B. Longitudinal section of wheat after treatment with phloroglucin and hydrochloric acid. Walls of cross layer cells (1) which contain lignin are stained red and appear dark. Epidermis which has washed away appears at 2. $\times 200$.

preliminary treatment. After treatment with potassium hydroxide in glycerol, the walls of the epidermis cells (Fig. 6A) and the wheat hairs (Fig. 6B) reacted strongly with the reagent.

Pectic Compounds. Treatment of cross and longitudinal sections of the wheat kernel with ruthenium red indicated that pectic com-

pounds occurred in the walls of all of the bran layers (Fig. 7A). The cell walls of the epidermis layers in flat strip sections were stained with this reagent; however, after treatment with hot dilute hydrochloric acid and hot potassium hydroxide solutions the brush and butt ends of the epidermis still stained. The walls of the cross layer and the tube cells were stained by ruthenium red (Fig. 8). The walls of white wheat testa cells (Fig. 7B) and the middle lamellae of the hyaline and aleurone cells stained faintly with this reagent. The interior of the aleurone cells stained strongly (Fig. 9A).

Lignin. As indicated by the pink color reaction given by the phloroglucin-hydrochloric acid reagent when the test is applied to



Fig. 10. A. Wheat hairs after treatment with phloroglucin and hydrochloric acid. Lignified areas are pink and appear dark. $\times 900$.
B. Cross section of wheat after staining with Sudan IV. Testa (1) is apparently cutinized and is stained red, appearing dark in photograph. Aleurone cell contents (2) also stain due to their fat content. $\times 200$.

cross and longitudinal sections of the wheat kernel, lignin occurs in the cross layer cells, particularly in that portion adjacent to the testa cells (Fig. 9B). This observation was verified by making the test on a flat strip section of cross cells in which lignin appeared to be located either in the outer part of the cell wall or in the middle lamella. Lignin also occurred in the walls of the wheat hairs (Fig. 10A) and in the epidermis at the butt end of the grain. Some samples of epidermis contained scattered areas of lignification other than at the ends of the kernel. These consisted of lignified walls of a small group of cells, isolated cells, or, rarely, partial lignification of the cell wall of an isolated cell.

Cutin and Suberin. Cutin occurred as a covering of the epidermis, as indicated in cross or longitudinal sections of the wheat kernel under

the polarizing microscope (Fig. 1B). A positive ceric acid test was obtained upon epidermis in a strip section.

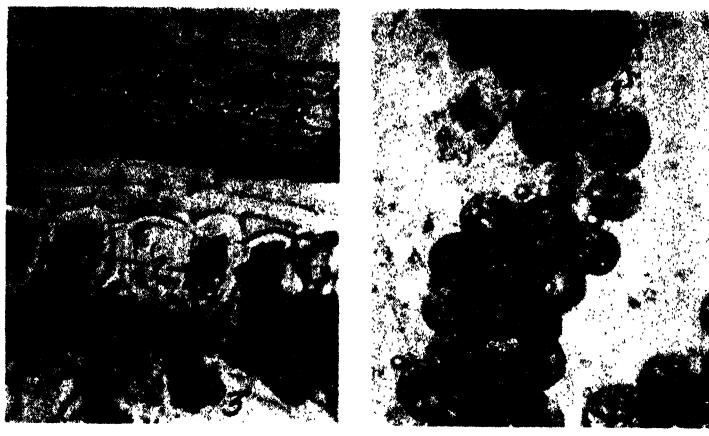


Fig. 11. A. Cross section of wheat after execution of xanthoproteic test. Testa (1), aleurone cell contents (2), and starchy endosperm (3) all react strongly and appear dark in photograph. $\times 200$. B. Flat section of aleurone cells (1) superimposed upon hyaline cells after execution of xanthoproteic test. $\times 200$.

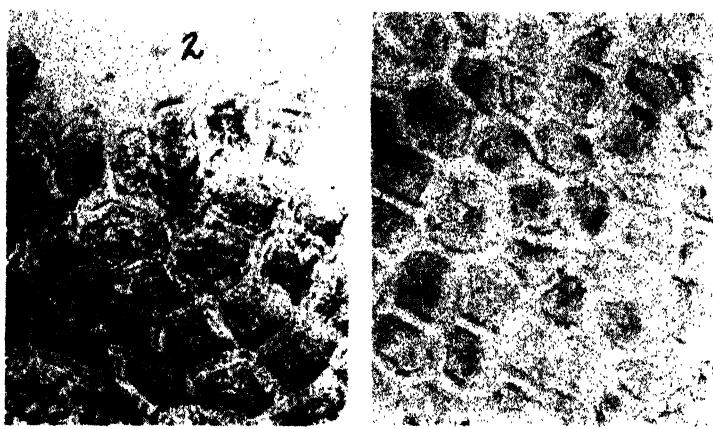


Fig. 12. A. Photomicrograph of flat section of aleurone (1) superimposed upon hyaline cells (2) after applying picric acid test. Tissue containing protein is stained yellow and appears dark in photograph. $\times 200$. B. Flat section of aleurone to which biuret test has been applied. $\times 200$.

Staining a cross section of the wheat kernel with Sudan IV indicated that the testa was either suberized or cutinized (Fig. 10B). With this dye the contents of the aleurone cells also stained very strongly. A strip section of testa removed by alcoholic sodium hydroxide solution

was also stained with Sudan IV. When a strip of whole bran was treated with 75% sulfuric acid solution for several minutes and then washed with water to remove the cellulose, pectin, pentosans, and proteins, the outlines of the testa cells could still be distinguished in the residue. This bran residue stained readily with Sudan IV and gave a positive ceric test. However, potassium phellonate tests were negative.

Proteins. As indicated by the xanthoproteic test on cross and longitudinal sections of white wheat, the proteins of bran occur largely in the testa layers (Fig. 11A). A small amount is found in the cross layers and the epidermis. A strip section of aleurone and hyaline, when treated with the xanthoproteic reagents, reacted only in the interior of the aleurone cells (Fig. 11B). Similar results were obtained using the picric acid test (Fig. 12A) and the biuret reaction (Fig. 12B). The starchy endosperm also gave a strong test for protein by all three methods.

Discussion

From the reactions with chloro-zinc-iodide it appears that cellulose occurs in abundance in the epidermis, the cross layers, the tube cells, and the hyaline cells. It is present to some extent in the cell walls of the aleurone, but is almost totally absent in the testa layer. These results are in accord with the quantitative work on wheat testa (10).

Since the cell walls of the epidermis after the removal of cutin are anisotropic with positive elongation and since this anisotropy is lost after treatment with ammoniacal cupric hydroxide solution, it appears that the cellulose of the epidermis is laid down parallel to the long axis of the cell. This is also true of the wheat hairs and of the hyaline cells. On the other hand, the inner cross layer cell walls exhibit negative elongation in the strip section and in the cross sections of the kernel. In a longitudinal section of the kernel in which the cross layer cells themselves are in cross section the cell walls exhibit positive elongation. Apparently then, the cellulose in the cross layer cells is laid down perpendicular to the long axis of the cells or parallel to the long axis of the grain. The difference in optical properties of the outer cell walls and middle lamellae of the cross layer cells as compared to the inner ones may indicate the presence of cutin in these tissues. This is further indicated by loss of these properties upon treatment with glycerol-potassium hydroxide.

The isotropy of the testa layer is striking since it has been reported that suberin lamellae are anisotropic while cutin lamellae are not, and other tests indicate that the testa contains either cutin or suberin. The testa layer is still isotropic after treatment with 75% sulfuric acid solution which removes everything except cutin, suberin, chitin, or

phytomelane. Although Eckerson (2) reported that the nucellus of wheat was suberized, her tests could hardly have distinguished between suberin and cutin. Since a potassium phellonic test was not obtained and since the testa layer was isotropic, it appears that this layer is cutinized rather than suberized.

The pectic compounds, as shown by staining with ruthenium red solution, were quite generally distributed in the bran. The staining of the epidermis with ruthenium red after treatment with weak hydrochloric acid and sodium hydroxide solutions was probably due to lignin, but no further attempt was made to learn whether pectic compounds also occurred in the same areas. The cross layer cells stained with ruthenium red in the cell walls next to the middle lamella, but not in the middle lamella itself. The middle lamella of these cells apparently contains lignin instead of pectic compounds. The cause of the staining of the aleurone cell contents with ruthenium red was not established.

Tests for protein indicated that little protein is found in any of the bran layers with the exception of the testa layer (Fig. 11A). This is in agreement with the quantitative data. The protein of the aleurone occurs almost entirely within the interior of the cell (Figs. 11B and 12).

Acknowledgment

The author is indebted to Dr. H. C. Sampson, Professor of Botany, Ohio State University, for his instruction and aid in the use of the microchemical methods employed in this work.

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STUDY OF THE WATER CONTENT OF SINGLE KERNELS OF WHEAT¹

T. A. OXLEY²

ABSTRACT

Apparatus and technique are described which are suitable for the determination of the water content of single kernels of wheat in lots of 40 kernels at a time.

The standard deviation of each set of 40 water content results may be used to measure the range in water content. Wheat which has been stored in small containers (i.e., of the order of one liter or less) has a standard deviation in water content ranging from 0.13 to 0.41%.

Distinction is made between a range of water content due to kernels being in equilibrium with different humidities (the "unsteady state") and a range due to physical differences in the kernels giving differences in water content when all are in equilibrium with the same relative humidity (the "equilibrium state").

The range of water content is temporarily increased by exposure of wheat to very dry or damp atmospheres which lead to rapid drying or damping of the kernels. This is evidence that the kernels differ in the rate at which they are able to exchange water vapor with the atmosphere.

In one case investigated (using a sample of a soft white Scandinavian variety "Als"), rapid drying produced a skew distribution of water content. This is evidence of skew distribution among the kernels of a character which affects the rate at which they exchange water vapor with the atmosphere. There are indications that this is also true of some other varieties studied.

Wheats of mixed origin were generally found to have a wider water content range in the equilibrium state than wheats derived from a single crop.

In a single study of a soft red wheat (Squarehead Masters), ears during ripening were found in general to dry out first at the apex and last at the base, although a few kernels near the base of an ear dried as quickly as apical kernels. The range of water content within a single "dead ripe" ear was found to be large (standard deviation 0.74), but this was probably partly an unsteady state due to incomplete drying out.

The water content of a sample of grain may be determined with some precision, but since the kernels of the sample vary among themselves in size, shape, consistency, and degree of maturity at harvest, it is reasonable to expect that there may be differences in their water content even though they have lain together in the same container for a considerable period.

It was thought desirable to investigate the range of water content exhibited by grains of various types and histories for three reasons:

- (1) If the range of water content is known it is possible to evaluate the

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error in water content determination which will follow from this source of variation in samples of various sizes. (2) Study of the water content of single kernels provides an indirect method for investigation of the way in which individual kernels exchange water with the atmosphere. (3) It was thought that differences in water content between individual kernels might have an influence on the choice of grains for oviposition by grain weevils (*Calandra* spp.). No evidence bearing on the third point has been obtained during the present investigation.

Apparatus and Methods

The first investigations were carried out by crushing the kernels and drying them in small labeled glass or metal containers in the hot air oven which is used for normal determinations in the laboratory. This method was abandoned because it was excessively laborious and the water losses during crushing of the kernels were found to be quite serious. Instead of this a method was developed in which the kernels were not broken or cut but were dried whole.

Determination of water content on whole kernels has three distinct advantages: (1) There is no danger of loss of water vapor or material during crushing or grinding. (2) The labor of crushing or grinding (with the need for cleaning a small crushing or grinding apparatus between each kernel) is obviated. (3) The rate of water vapor exchange between whole kernels and the atmosphere is much slower than that of crushed or ground kernels. For this reason it is possible to weigh whole kernels, even when freshly dried, direct on the scale pan without appreciable error due to gain or loss of water vapor provided that the period of exposure does not exceed 30-40 seconds.

These advantages are very real in so far as they reduce labor, for a statistical study of the range of water content requires many determinations from each sample. The standard number of kernels determined per sample for the present investigation was 40 and the labor involved in these determinations would have been prohibitive if all laborsaving techniques had not been adopted.

The disadvantage of using whole kernels is that the rate of loss of the last traces of water is excessively slow. It was desired to use a temperature not greatly different from that which is standardized in this laboratory for normal water content determination (115°C.), and it was found that satisfactory results with whole kernels could only be obtained at or near this temperature, by use of a vacuum desiccator and a period of at least 48 hours. The apparatus described below was therefore constructed and, having adopted for convenience a standard drying time of 48 hours, the temperature was varied until agreement was obtained between the mean water content of 40 whole

kernels and the water content of a sample of grain from the same sealed bottle determined by the standard laboratory technique.³ Agreement was satisfactory when the temperature of the surface on which the kernels lay was 120°–125°C.

Drying Apparatus. The arrangement of the vacuum desiccator used for drying 40 whole kernels simultaneously is shown in section in Fig. 1.

It was found very inconvenient to have a heater within the desiccator supplied by leads through the lid and accordingly a system of induction heating was adopted. A bundle of $\frac{1}{4}$ -inch diameter soft iron rods stands vertically in the center of the desiccator and serves as a magnetic core in which an alternating magnetic field is induced by the exciting coil. The latter (which consists of 12 lbs. of copper wire 0.071 cm. diameter) is wound on a wooden former and fixed to the wooden stand in which the desiccator rests loosely.

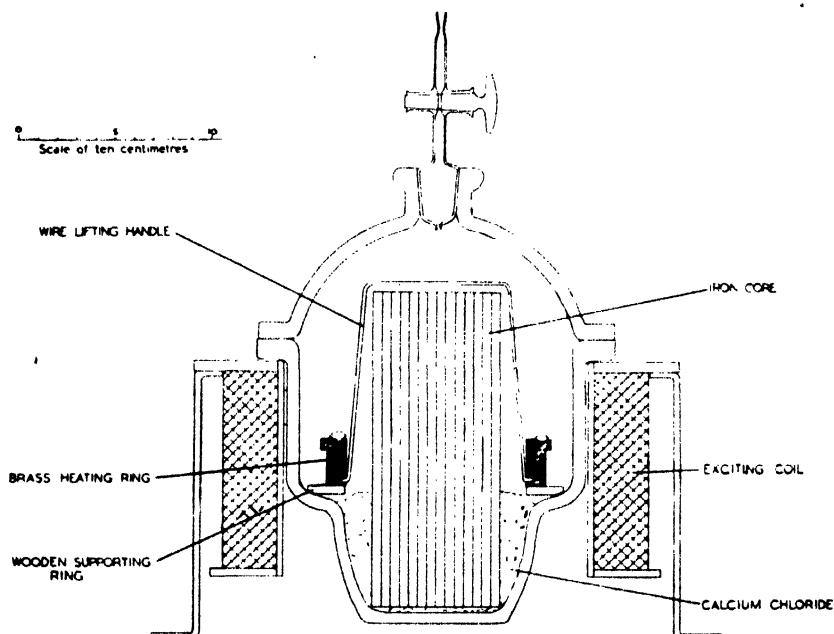


Fig. 1. Vertical median section through drying apparatus.
Thermometer is not shown in the drawing.

The heater consists of a brass ring, of the form shown in Fig. 1, which surrounds the central iron core at about its mid height. The alternating current that is induced in this ring produces sufficient heat to raise it to the desired temperature. The amount of heat dissipated

³ Grain coarsely ground and dried for 4 to 4½ hours in a ventilated oven at 115°C.

in this ring, and hence the temperature which it reaches, can be varied by varying the voltage applied to the exciting coil. This adjustment is made by means of a tapped auto-transformer not shown in the diagram. The voltage used is 322 at 50 cycles per second.

The temperature of the ring is indicated by a thermometer whose bulb rests in a hole bored vertically in the ring. Thermostatic control is not applied to the ring and hence the temperature reached depends partly on the temperature of the room in which it is placed. In practice the variations are slight (the extremes being of the order of $\pm 5^{\circ}\text{C}.$) and no effect on the water content results has been observed. Even if slight variations of the mean water content determined by this method do occur they are unimportant for the present investigation, since the aim is to study interkernel variations rather than absolute water content values.

Forty shallow conical depressions about 7 mm. diameter and 1.5 mm. deep and numbered 1-40 are machined in the upper surface of the ring to receive the kernels for drying. The ring is provided with a wire handle (which runs parallel with the magnetic field and therefore does not become hot) and by this means may be conveniently transported to and from the balance room so as to receive kernels directly after weighing.

At the end of a drying period the lid of the drying chamber is removed and, while the ring is still hot, the kernels are transferred as quickly as possible to 40 small, numbered desiccators. Each of these consists of a glass tube 30 mm. long and 6 mm. internal diameter sealed at one end and closed with a rubber stopper at the other. A small amount of phosphorus pentoxide is put at the bottom of the tube and covered with a pad of cotton wool on which the kernel rests. Kernels cool rapidly in the desiccators and may be weighed immediately, but there is no gain in weight if they are left for several days before weighing.

In addition to the desiccators there are several sets of smaller rubber-stoppered glass bottles 15 mm. long by 4.5 mm. internal diameter which contain no drying agent. These are used for isolating sets of 40 kernels at various stages in the wetting and drying experiments or for collecting samples from various sources. Once isolated in these tubes kernels do not change in weight over periods of many days.

The Balance. It was desired to measure water contents of kernels with an accuracy of $\pm 0.05\%$. Since the usual weight of kernels employed in the present investigation was between 20 and 50 mg., this required that weighings should be accurate to ± 0.01 mg. It is not usually possible to combine this degree of accuracy with a rapidity

of operation such that the period of exposure of the kernels does not exceed 40 seconds. This difficulty was overcome by the use of a special balance designed and manufactured by L. Oertling and Sons, Ltd.

This consists of a microbalance with an optically projected scale for reading the last two decimal places such as is standard in this firm's normal commercial model. To this instrument the following modifications are made in order to satisfy the requirements of rapid weighing and accuracy specified above: (1) The sensitivity is reduced to one-tenth of the normal (i.e., full scale deflection represents 1 mg.). (2) Air damping is fitted so as to make the balance aperiodic. (3) Automatic loading is provided by means of four ring-riders. This enables loading by 10-mg. intervals from 0-90 mg. to be made by rotating a dial. (4) The usual 5 mg. rider is used only on every tenth notch of the notched beam, thereby giving loading from 0-9 mg. by 1-mg. intervals.

Judgment by eye of the weight of a kernel to within 10 mg. is easy. With practice it is possible to weigh most kernels with only one or two movements of the rider and the balance can thus be released within 15 seconds of the kernel being dropped on to the pan from its bottle or desiccator. The beam requires 15 seconds to come to rest after release and thus the whole weighing can be completed within 30-40 seconds.

Estimation of Errors. Since no independent check on the water content of a single kernel is possible, all errors appear as interkernel variations. Hence it is particularly important to examine the sources of error. There will probably be some variations in the extent to which the loss of weight on drying under particular conditions represents the "true" water content as it might be defined in physico-chemical terms. But such variations, which may be called the "intrinsic" variations, are inherent in all known methods of water content determination and it is at present impossible to evaluate them. Hence, except in terms of an abstract physicochemical definition, it has no precise meaning to speak of a "true" water content. The term water content can thus only have definite meaning if the precise method of determination is specified.

The indirect loss-of-weight-on-drying method was adopted for the present investigation because it is the method most easily adapted to determination of single kernel water content. It is an advantage of the choice that similar methods are probably the most widespread throughout the world in laboratory and trade practice with cereals. The present results are therefore probably subject to the same intrinsic sources of error as all similar methods.

What may be called the extrinsic sources of error are those which arise from the technique, producing differences between the loss of weight on drying under constant conditions and the estimate of them. The possible sources of such errors in the present technique appear to be: (1) Human errors. (2) Variations in conditions between the numbered places occupied by the kernels during the course of the determination, i.e., variations in ring temperature from place to place and variation in humidity in the desiccators. (3) Errors in weighing.

It is believed that the technique leaves very little room for human errors to remain undetected. Unevenness in the temperature of the ring would produce an error, but such an error should be consistent over a long period since a difference in temperature between one part of the ring and other parts could only arise from a defect in the brass casting which caused local variations in electrical resistance. A similar consistent error would arise if any fault (such as exhaustion of the desiccant) existed in one of the single kernel desiccators. In order to ascertain whether any such consistent errors existed part of the data was examined in the following way.

A number of sets of water content determination records were chosen. For this purpose only sets having a standard deviation less than 0.3 were used since variability greater than this occurred only when the kernel had been subjected to a wetting or drying treatment in which cases it is clear that the excess variability is certainly in the material and is not characteristic of the technique. Thirty such sets were available. Thirty water content percentages obtained in each of the 40 positions were added together and the variance of the 40 totals was calculated and found to be 2.292. Therefore the variance between single determinations is estimated to be $\frac{2.292}{30 \times 30} = 0.00255$.

It has been assumed that this variance between single determinations is due solely to consistent inequalities in the technique related to the numerical position of the kernels. It is certainly not an underestimate and is probably an overestimate, since with so small a number as 30 sets some of the variance must be due to random distribution of variations in the material.

Errors in weighing are probably the most serious extrinsic source of variability. In order to estimate these, 40 kernels contained in 40 of the rubber-stoppered bottles described above were weighed seven times over a period of 9 days. In each case the kernels were taken in random order so as to avoid the danger that the operator might learn the weights. The weighings were thus conducted under precisely the same conditions as were the weighings for determination of water content.

This process provided seven estimates of the weight of each of the 40 kernels. For each kernel, the sum of squares of deviations from the mean was calculated. Allowing six degrees of freedom to this, the total sum of squares for the 40 kernels has 240 degrees of freedom. The variance of a single weighing calculated on this basis was found to be 0.000125 (all weighings in milligrams and decimals of a milligram).

The total weight of the 40 kernels showed a small progressive decrease which was first detectable on the fifth total. This was presumably due to slight drying out of the kernels during their successive exposures to the atmosphere during weighing. No allowance was made for this source of error in the above calculation of the weighing variance which is therefore presumably an overestimate.

The effect of the weighing error on the water content estimate varies according to the size of the kernel. If x = wet weight of the kernel in milligrams, and y = the dry weight,

$$\text{variance of } \frac{x - y}{x} = \frac{\text{var. } x + \text{var. } y}{x^2}$$

$$\text{but variance } x = \text{variance } y = 0.000125.$$

Therefore, variance of water content estimate: per cent of wet weight

$$= \frac{100^2}{x^2} \times 2(0.000125) = \frac{2.5}{x^2}$$

Therefore, if the weight of a kernel is as low as 15 mg. (which is the lowest extreme used in the present experiments) the variance in water content estimate due to weighing error is $\frac{2.5}{15 \times 15} = 0.011$. For heavier kernels it is clear that the variance due to this cause is very much less and is quite negligible by comparison with the variances of the water content estimates of the material.

In Table I are presented data from samples showing the minimum of variability, in which the weighing error and "place" error are of the greatest importance. In this table the variances due to these causes are given (that for weighing being calculated on the basis of the mean kernel weight) and in the last column the standard deviation which remains after these two variances have been subtracted is given. It will be seen that even these data are scarcely affected by these sources of error (compare columns 5 and 11). In all later data no allowance is made for errors since they are negligible by comparison with the variability of the material. It is clear that the variabilities studied in the present work are true variabilities of the material.

Results and Discussion

Differences in water content between kernels can be of two kinds: (1) Kernels may so differ physically that their water-holding capacities differ when in equilibrium with a given atmospheric relative humidity. This is referred to below as the "equilibrium state." (2) Kernels may be in equilibrium with different atmospheric relative humidities. This state of a sample is referred to below as the "unsteady state."

In the unsteady state the kernels are exerting different water vapor pressures, and if such kernels are contained in a common container their water contents will be unsteady; they will exchange water by diffusion until all kernels in the container exert the same vapor pressure. They will then be in equilibrium with the same relative humidity and the residual differences in water content will be of the first kind, i.e., the sample will be in the equilibrium state. The time required for such equilibration must depend very largely on the size of the container. No adequate data exist on this point, but some of the present data give evidence relating to very small containers.

Diversity in the Equilibrium State. Differences in water content in the equilibrium state appear to be distributed approximately according to a normal frequency distribution. Hence it is convenient to use the standard deviation of the results as a measure of the range of water content. Determinations have been made on a number of samples of wheat which, whatever their previous history, had been stored in the laboratory in small sealed bottles (about 200 ml.) or rubber-stoppered test tubes (about 30 ml.) for periods varying between a month and a year. It is assumed that relative humidity equilibrium had been completely attained in all these samples and that the variability remaining was due solely to physical differences in the kernels. The results of a series of such determinations are given in Table I.

It is clear from the data in Table I that there is an irreducible minimum of water content variation in wheat of various varieties when in equilibrium with the same relative humidity. The standard deviation of the various samples quoted is in the neighborhood of 0.2 based on samples of 40 kernels in each case.

It has not been possible to find any observable character in the individual kernels which could be correlated with their water content. On a number of occasions each kernel of a series has been carefully examined for such characteristics as color, wrinkledness of skin, length relative to breadth, plumpness, and freedom of the skin from breaks. No relation has been found between any of these and water content. There is also no sign of any correlation between water content and the weight of kernels taken from a sample in the equilibrium state. This fact is some evidence that the water content determination technique is

TABLE I

INTERKERNEL VARIABILITY OF WATER CONTENT IN SAMPLES OF VARIOUS VARIETIES OF WHEAT STORED IN SMALL QUANTITIES FOR ONE MONTH OR LONGER

Date	Variety	Water content mean	Gross variance	Stand-ard deviation	Error correction			Total ex-trinsic variance	Residual	
					Mean kernel weight	Weighting variance	Place variance		Variance	Stand-ard deviation
24.4.46	Atle	18.15	0.048	0.219	37.18	0.0018	0.0026	0.0044	0.044	0.210
30.4.46	Unnamed white winter	15.03	0.058	0.240	48.65	0.0011	0.0026	0.0037	0.054	0.232
1.7.46	Als	18.33	0.037	0.193	47.81	0.0011	0.0026	0.0037	0.033	0.182
10.8.46	Als	20.55	0.049	0.222	46.45	0.0012	0.0026	0.0038	0.045	0.212
4.2.47	"Manitoba"	13.97	0.063	0.252	31.43	0.0025	0.0026	0.0051	0.058	0.241
8.2.47	Extra Kolben	13.61	0.022	0.150	34.06	0.0022	0.0026	0.0048	0.017	0.130
4.3.47	Meteor	13.42	0.043	0.207	34.41	0.0021	0.0026	0.0047	0.038	0.195
7.3.47	Borsc	12.95	0.169	0.411	52.14	0.0009	0.0026	0.0035	0.165	0.406

unaffected by kernel size, i.e., large and small kernels are dried equally effectively.

Effect of Recent Change in Water Content on the Interkernel Variation.

It was observed that samples which had recently been dried by exposure to air of low humidity, or damped by exposure to air of high humidity, exhibited a wider diversity than those which had not been so treated. It was also observed that the increased diversity resulting from rapid gain or loss of water vapor disappeared during the course of a few days from a small sample confined in a sealed container, the diversity reverting to that characteristic of the equilibrium state. It was therefore concluded that the increased diversity was an unsteady state, the various kernels being in equilibrium with different humidities. It also follows that rapid drying or damping at ordinary temperatures does not affect the water-holding capacity of the kernels, since the diversity at equilibrium state was unchanged. The fact that diversity is increased by rapid change, however, shows that kernels differ in the ease with which they gain or lose water.

Kernel samples were dried by exposure for the stated period in a wire gauze basket in a desiccator containing calcium chloride and fitted with a fan in the lid. Samples were damped by spreading the kernels on a piece of perforated zinc held 1.5 mm. above a water surface in a petri dish. A filter paper soaked in water was fixed inside the lid about 8 mm. above the kernels. In the case of both drying and damping, the number of kernels seldom exceeded 200 and never formed a complete layer over the gauze or perforated zinc. It was considered

that all kernels had substantially equal exposure to the wet or dry atmosphere and hence substantially equal opportunities for water vapor exchange. The samples that were left for varying periods after treatment, before isolation in separate bottles, were always small, usually 100 to 150 kernels. This is an important factor, for larger samples in which the diffusion paths for water vapor might be larger would be expected to take longer to reach equilibrium. Data obtained are presented in Table II.

TABLE II

EFFECT OF RAPID EXCHANGE OF WATER VAPOR BETWEEN WHEAT KERNELS AND ATMOSPHERE ON WATER CONTENT DIVERSITY OF THE KERNELS¹

Wheat variety	Treatment	Period sealed for equilibration after treatment	Mean water content	Standard deviation
Als ²	Original sample	—	18.33	0.19
Als	Dried 0.2 hour	0	17.79	0.19
Als	Dried 1 hour	0	16.37	0.25
Als	Dried 2.5 hours	0	15.54	0.52
	Dried 2.5 hours	73	15.35	0.22
Als	Dried 5.5 hours	0	12.67	0.92
	Dried 5.5 hours	137	12.88	0.16
Als	Damped 4.8 hours	0	20.86	0.40
	Damped 4.8 hours	92	20.55	0.22
Manitoba ³	Original sample	—	13.88	0.27
Manitoba	Dried 6 hours	0	11.83	0.54
Manitoba	Damped 6 hours	0	19.44	0.75
	Damped 6 hours	168	19.60	0.18
Manitoba	Damped 6 hours	0	19.44	0.44
	Damped 6 hours	6	19.30	0.35
	Damped 6 hours	18	19.08	0.30
	Damped 6 hours	260	19.41	0.17

¹ Table also shows subsequent decrease in water content diversity as result of storage for varying periods in a sealed container.

² Als is a soft white winter wheat of Scandinavian origin.

³ "Manitoba" wheat is a sample of the commercial grade No. 1 Manitoba wheat as exported from Canada. It probably consisted entirely of Thatcher and related hard spring wheats.

It is noticeable that the two samples of Manitoba wheat which were damped for 6 hours differ considerably in the amount of diversity which this treatment produced; in one case the standard deviation is 0.75, in the other 0.44. This failure of apparently identical treatments to produce similar amounts of diversity has been observed several times.

Distribution and Nature of the Factor Which Controls Rate of Water

Vapor Exchange. The increase in diversity due to rapid exchange of water vapor shows that the rate of water vapor exchange depends partly on a factor which is not equal for all kernels. If the values for this factor are normally distributed among the kernels, the water content frequency distribution will remain normal after a damping or drying treatment, even though the standard deviation is increased.

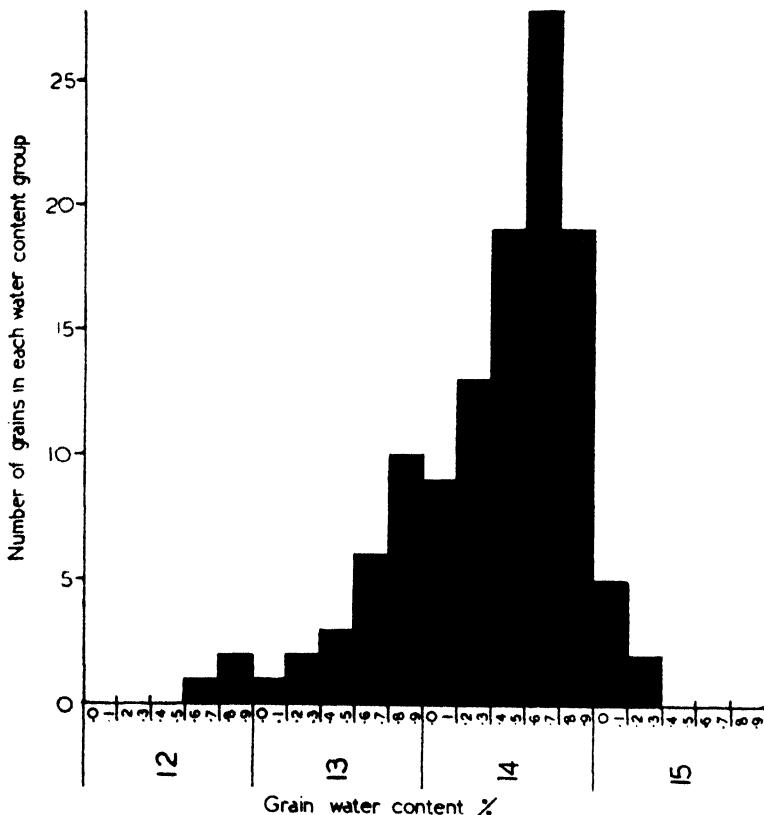


Fig. 2. Distribution of water content in 120 kernels of wheat (variety "Als") immediately after drying for 6 hours at room temperature. Initial water content was 18.3% and standard deviation 0.19; final mean water content was 14.4% and standard deviation 0.515. Skew distribution, which results from rapid drying, is well shown.

Examination of the data shows that the water content frequency distribution often becomes skew as a result of a damping or drying treatment which implies that the distribution of the factor controlling water vapor exchange is itself often skew.

Rapid drying of the soft wheat "Als" produces a skew distribution curve whose bias is towards the higher water contents. This is illustrated by the results shown graphically in Fig. 2. With initial water content 18.3% and standard deviation 0.19, 120 kernels of Als were

dried for 6 hours at room temperature in the desiccator with a fan, thereby reducing the mean water content to 14.40% and increasing the standard deviation to 0.515. The skewness of the distribution, with its bias towards the higher water contents, is fairly clear from Fig. 2. The prolonged "tail" towards low water contents indicates that there is a small proportion of kernels which lose water more rapidly than the majority. The distribution is skew because there is not a similar proportion of kernels which lose water much more slowly than the majority.

This is the only set of data available for a large number of kernels. The remaining relevant data (some of which are shown in Fig. 3) consist of 40-kernel sets which are barely large enough to show this effect. Fig. 3A shows a further set of Als, dried for $5\frac{1}{2}$ hours, in which the skew distribution is apparent. Fig. 3B shows the effect of 6 days' storage in an airtight container of a portion of the same sample.

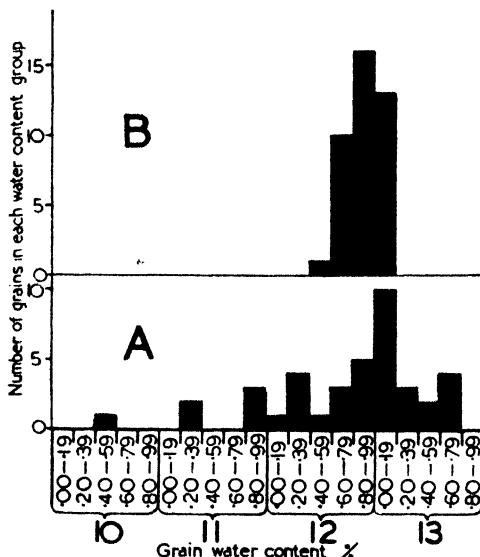


Fig. 3. Distribution of water content in 40 kernels of wheat (variety "Als"); (A) immediately after drying for $5\frac{1}{2}$ hours at room temperature; (B) after 6 days' storage in a small sealed container immediately subsequent to drying.

Investigation on these lines has not been extended further since it is apparent that the present technique is not well adapted for the purpose. Further investigations would be more fruitful if based on a study of the rates of water exchange of individual kernels.

Distribution of Water Content in Mixed Wheats. Samples were taken in a small country mill⁴ where English wheats from various

⁴ Coxes Lock Mill, Weybridge. The author is indebted to Sebert Humphries for permission to take the samples.

sources were at the time being mixed with Manitoba and Dark Hard Winter wheats to form the grist. The objects in taking these samples were: (1) To ascertain what range of water content actually existed in the grist entering the first break rolls. (2) To study the equilibration of the sample when sealed at room temperature in order to see whether widely different wheats would settle to considerably different water contents.

At the time of sampling, English wheat was being drawn from a silo bin which contained several lots from different sources. These lots formed consecutive layers in the bin (and presumably exchanged water very slowly) but became mixed during withdrawal of the grain from the bottom of the bin. On emerging from the bin, the wheat was briefly washed, whizzed, and dried. A second sample was taken immediately after this treatment. Mixed Manitoba and Dark Hard Winter wheats (which were not sampled) were not washed in any way but were mixed with the English wheat soon after the latter left the drier. The mixture was made in the proportion 65% Manitoba and Dark Hard Winter to 35% English. The third sample was of this mixed grist, which had probably lain in the grinding bins for only a few minutes, and was taken at the point of entry to the first break rolls.

Each sample consisted of 40 kernels, which were immediately isolated in separate glass tubes, and a 30 ml. mass sample which was tightly sealed. Lots of 40 kernels were withdrawn from these from time to time for study of the equilibration process.

The results of these water content determinations are shown graphically in Fig. 4. Fig. 4A (untreated English wheat) shows the exceedingly wide range of water content (standard deviation = 1.266) which results from the mixing of small lots in the intake bins during the course of drawing off. Fig. 4D shows that washing and slight drying have not reduced this range (standard deviation 1.534). Fig. 4G shows the result of mixing 35% of the very wide range English wheat with double the quantity of the (presumably) much more homogeneous Dark Hard Winter and Manitoba mixture (standard deviation 1.222). This mixture is the grist taken as it entered the first break rolls.

The successive stages of equilibration of these samples in 30 ml. lots at room temperature are shown in Fig. 4B and 4C, 4E and 4F, and 4H, J, and K. Two facts emerge from a study of these: (1) Equilibration is very slow—even at room temperature (15°–25°C.) and in a very small container whose longest dimension was 10 cm. (2) When equilibration is presumably nearly complete the range of variation remains quite wide, a fact which reflects the varied origin of the wheats.

This is most evident in Fig. 4K where the mixed grist after 51 days shows a wide and skew distribution; presumably the skewness is due to the unequal mixture of the hard and soft wheats which appear to retain low and high water-holding capacities respectively.

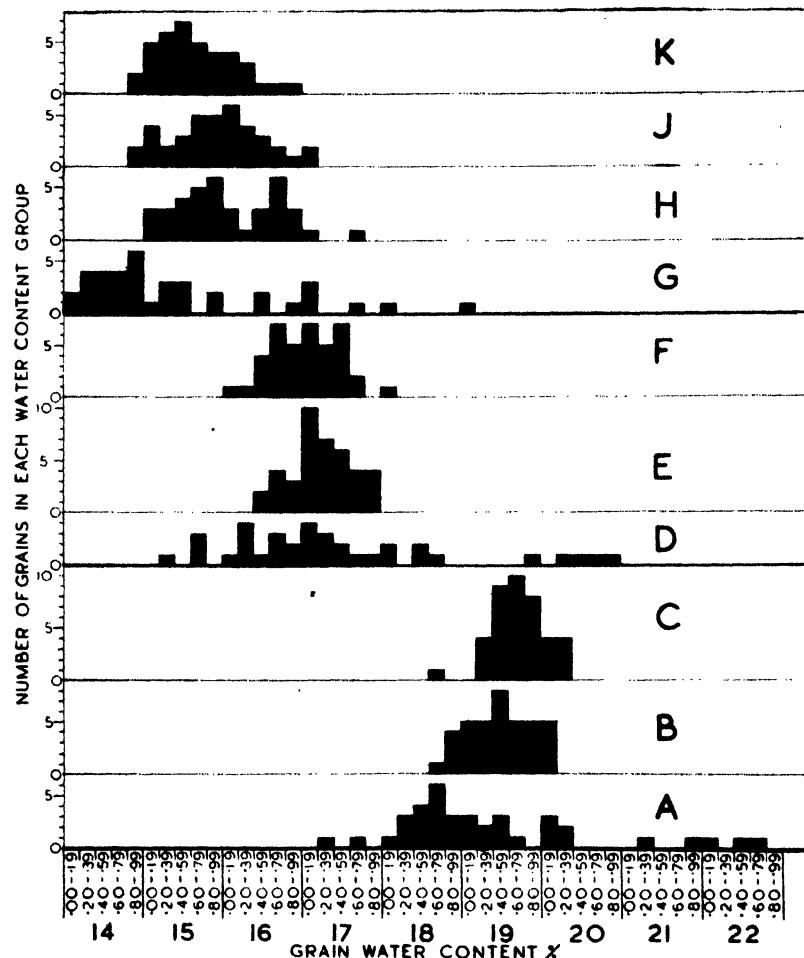


Fig. 4. Distribution of water content in mixed wheats at a country flour mill: (A) English wheat as drawn from the intake bins which had been filled with wheat from various sources. (B and C) Subsequent equilibration of the sample after 19 and 32 days respectively. (D) Mixed English wheat from the intake bins after washing, whizzing, and partial drying. (E and F) Subsequent equilibration of the sample after 19 and 32 days respectively. (G) Mixed grist (65% "Manitoba" and Dark Hard Winter wheats and 35% English) entering the first break rolls. (H, J, and K) Subsequent equilibration of the sample after 19, 32, and 51 days respectively.

Water Content Variations within a Single Ear. In July and August, 1944, a single ear was picked at random from a field of wheat (variety Squarehead Masters) on each of five occasions during the ripening

TABLE III

WATER CONTENT OF KERNELS OF EARS OF WHEAT PICKED AT RANDOM
ON FIVE DATES FROM A SINGLE FIELD¹

Kernel No.	Water content				
	1st ear July 20	2nd ear July 24	3rd ear August 1	4th ear August 3	5th ear August 8
1 ²	19.5	21.2	19.3	17.1	15.2
2	19.0	36.1	20.9	17.4	14.7
3	16.5	35.9	19.0	17.5	16.7
4	21.4	20.1	18.0	17.1	15.7
5	18.1	26.7	21.2	17.1	14.6
6	21.1	34.4	21.4	17.0	15.8
7	24.5	19.7	19.6	16.3	16.6
8	15.5	36.7	18.2	17.2	15.3
9	28.8	36.2	20.0	17.2	15.3
10	32.7	34.1	20.3	16.9	15.9
11	30.4	30.7	19.7	16.7	15.0
12	33.3	48.6	22.2	16.6	15.5
13	31.5	30.3	19.0	15.8	14.5
14	33.2	35.7	22.2	15.7	14.3
15	33.4	38.9	20.9	16.3	14.1
16	26.8	35.8	20.1	16.7	15.1
17	35.2	40.9	21.7	17.3	14.8
18	23.5	35.7	22.6	16.6	15.8
19	36.8	38.4	19.3	17.4	14.4
20	36.8	38.1	21.1	16.8	15.0
21	32.6	23.3	23.5	16.5	15.7
22	39.1	38.1	23.0	17.6	14.1
23	37.3	33.0	19.0	17.3	14.1
24	25.1	38.1	21.8	17.9	14.3
25	39.7	21.4	21.0	17.7	14.9
26	32.9	34.2	23.5	18.3	15.4
27	25.9	43.7	23.7	16.8	15.2
28	40.8	34.8	22.3	17.6	15.0
29	23.9	42.3	24.0	18.6	15.7
30	40.1	36.0	21.2	18.0	15.0
31	32.8	42.4	23.6	18.0	14.0
32	39.1	41.5	20.1	17.7	14.9
33	39.9	42.8	22.6	17.6	14.9
34	41.5	39.7	22.7	19.0	15.1
35	31.9	42.8	27.0	17.9	16.1
36	41.1	43.1	23.7	19.1	16.2
37	37.1	40.8	23.5	18.8	14.8
38	—	40.4	24.9	—	14.5
39	—	42.5	21.4	—	15.2
40	—	42.8	20.4	—	17.1
Mean	30.78	35.95	21.49	17.33	15.16
Variance	60.62	49.93	3.86	0.651	0.548
Standard deviation	7.79	7.07	1.97	0.807	0.740

¹ Variety was Squarehead Master.² No. 1 equals apical kernel.

period. Kernels were removed from the ears one at a time, starting from the apex, and the water contents of these (or of the first 40 kernels if the ear contained more than 40) were determined. The results of these determinations are given in Table III.

The weather was generally dull and cloudy during the period concerned, but rain and night dew were slight and infrequent. Humidities were high for the time of year but fell during most of the period between picking of the fourth and fifth ears to a normal dry summer level.

From these data the following conclusions may be drawn: (1) For this variety of wheat, in the climate of the Thames Valley in July and August, the apex of the ear tends to dry out first, though a few lower kernels may be as far advanced in drying as the apical kernels. (2) The range of variation within a single ear is very great indeed before drying out is complete. The range decreases as drying out progresses but is still much greater than is found in wheat of a single crop after storage for a few months. Presumably the range observed within a single ear represents an unsteady state, i.e., it is due to some kernels being not yet in equilibrium with the atmosphere even though the ear taken on August 8 was judged dead ripe.

The Effect of Variation in Intercernel Water Content on Desirable Size of Sample for Water Content Determination. The fact that all kernels in a sample taken for determination of water content do not have the same water content introduces an error into the determination. If the sample contains "n" kernels and the standard deviation of interkernel water content variation is σ , the standard error introduced by this source of variation will be $\frac{\sigma}{\sqrt{n}}$.

It is usual to expect that the result of a regular water content determination shall be accurate to the nearest 0.1%. If the probability of being outside this limit is to be not greater than one in one thousand times, the standard error must be not greater than $\frac{0.1}{3.29} = 0.0304$. The effect of interkernel variation on the desirable minimum sample size to insure a standard error not greater than this is given in Table IV. In the third column is given the size of the minimum sample in grams, assuming a mean kernel weight of 40 mg.

Thus a sample of 4 g. is sufficient to avoid error from this source if the grain sample is of homogeneous origin and has been stored for a few months (standard deviation up to 0.3). If the grain is of heterogeneous origin, from several fields or farms, but is well mixed and has been stored for a month or two (standard deviation up to 0.6) a sample up to 16 g. may be required. If the origin is heterogeneous and

TABLE IV
MINIMUM NUMBER OF KERNELS TO KEEP STANDARD ERROR OF
MEAN BELOW 0.0304%

Standard deviation	Minimum number of kernels in sample	Weight of sample, assuming mean kernel weight of 40 mg.
0.2	44	1.7
0.3	98	3.9
0.4	174	7.0
0.5	272	10.9
0.6	392	15.7
0.7	533	21.3
0.8	696	27.8
0.9	881	35.3
1.0	1087	43.5
1.5	2440	97.6

mixing has occurred only a few hours before sampling, the sample may need to be as large as 50 g. and in extreme cases up to 100 g. may be required.

Acknowledgment

The author is indebted to Dr. F. Garwood, of the Road Research Laboratory, for advice on the statistical treatment of the sources of error.

A MODIFIED AMYLOGRAPH METHOD FOR THE RAPID DETERMINATION OF FLOUR AMYLASE ACTIVITY¹

L. F. MARNETT, R. W. SELMAN,
and R. J. SUMNER²

ABSTRACT

A modified method is described wherein the amylograph is utilized for the rapid determination of amylase activity by measurement of flour paste viscosity. The procedure consists essentially of employing a starting temperature of 45°C. and a temperature rise of 3.5° to 4°C. per minute, which enables a single determination to be completed in 12 minutes or less. The method is highly correlated with the standard amylograph method now in use. General principles are discussed for the possible design of a simple, inexpensive, and rugged instrument to suit the requirements of the method described.

As the evaluation of amylase activity of bakery flours by physical measurements of paste viscosity becomes more extensively used, it has become increasingly apparent that, for application to routine control, the instruments now available for such measurements are too fragile,

¹ Manuscript received October 7, 1947; presented at the Annual Meeting, May, 1947. Contribution from C. J. Patterson Company, Kansas City, Missouri.

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too time-consuming, and too expensive. These instruments, which are described by Anker and Geddes (1), Brown and Harrel (3), and Bechtel (2), are designed primarily as research tools and as such are constructed to provide a versatility of application which is not necessary in specific routine control work.

A series of investigations was initiated to determine whether or not a more rapid procedure for determining peak viscosity with the amylograph would give results which are correlated with the present standard method.

Experimental

The conventional methods of measurement of the effect of malt upon the physical properties of flour pastes involve (1) a period of incubation during which the temperature of the suspension is increased from about 25°C. to the gelatinization range, (2) a period of gelatinization during which the temperature continues to increase. The maximum paste consistency obtained during this gelatinization is used as a criterion of the amylase activity of the flour.

To evaluate the relative importance of the various steps customarily employed in the determination of amylase activity of flour by this physical method, the effect of various incubation periods over a series of temperature ranges was studied by means of the amylograph. The results of an experiment in which two flours were submitted to amylograph analysis by the method of Selman and Sumner (4), and were then submitted to a procedure whereby the suspensions were made up at 45°C. and were gelatinized in the amylograph, the thermoregulator of which was started at 45°C., are shown below:

Sample number	Paste viscosity—(amylograph units)	
	Starting temperature	Starting temperature
	25°C.	45°C.
1	940	830
2	480	420

The results showed that a less viscous paste is formed by the latter procedure. This means that either more enzymic breakdown of the starch takes place when a large part of the incubation period is eliminated, or else that the incubation period provides an effect which increases the potential paste viscosity of the flour suspension. If there is thermal inactivation of the enzyme system during the incubation period, the former supposition may be true, although the indicated difference in enzyme activity is about 10%. The above findings were observed when using flour suspensions of 22.2% concentration and are

contrary to those of Anker and Geddes (1) who, when using 9.1% wheat starch suspensions, found that maximum paste viscosity increased with increases in starting temperatures exceeding 25°C.

The results would appear to indicate that there is virtually no enzyme action affecting the gelatinization characteristics of the suspension until the starch has started to gelatinize. The greater part of the starch conditioning effect must then take place between the temperature of the beginning of gelatinization and the temperature at which maximum viscosity is reached. It then becomes apparent that the only requirements which must be met in order to apply the method of measurement of paste characteristics to routine malt control are (1) that the suspension be heated from the gelatinization point to the point of maximum consistency at a reproducible rate which will vary inversely with the sensitivity desired and (2) that the consistency of the paste be continuously measured during this process by a system or device which will permanently indicate the maximum consistency reached.

To obtain some background information on the potentialities of these findings with respect to the speed of determination and its relationship to sensitivity, the amylograph was used with the following procedure:

Eighty-five grams of flour (weighed on a 14% moisture basis) were suspended in 455 ml. of dilute citrate-phosphate buffer (ph 5.3)⁸ at a temperature of 45°C. The suspension was placed in an amylograph the bowl of which was already warm. The amylograph thermoregulator, which had been previously set at 45°C., was manipulated manually at twice the normal rate of drive. This operation was continued until the flour suspension had reached its maximum gel consistency as indicated by the amylograph recording device. Ten to 12 minutes were required to reach this point. With this particular instrument, such a procedure produced a temperature increase of 3.5° to 4°C. per minute, depending upon the line voltage at the time of determination, since the heating system of the instrument was taxed to maximum capacity by this rate of temperature increase.

The reproducibility of maximum consistency obtained by this procedure was excellent if the line voltage was constant. The degree of variation of line voltage encountered in one day was sufficient to introduce an error of plus or minus 30 units, including the manipulation error of the instrument, since the enzyme effect, of course, varied inversely with the rate of temperature increase, whereas the rate of temperature increase varied approximately directly with the square of the voltage.

⁸ The composition of this buffer is citric acid 0.105% and dibasic sodium phosphate 0.148%.

In evaluating the practicability of the principles involved, this variation of enzyme activity with line voltage is not an inherent source of error, since any instrument specifically designed for this purpose would require a heating capacity adequate to provide a surplus over that required to produce the desired rate of temperature increase.

When increments of malted barley flour were added to several samples of unmalted flour, and the samples submitted to the above analytical procedure, the maximum consistency obtained varied inversely with the quantity of malt added, according to the relationship demonstrated in Fig. 1. The variation of apparent paste viscosity with malt concentration is sensitive enough for control purposes.

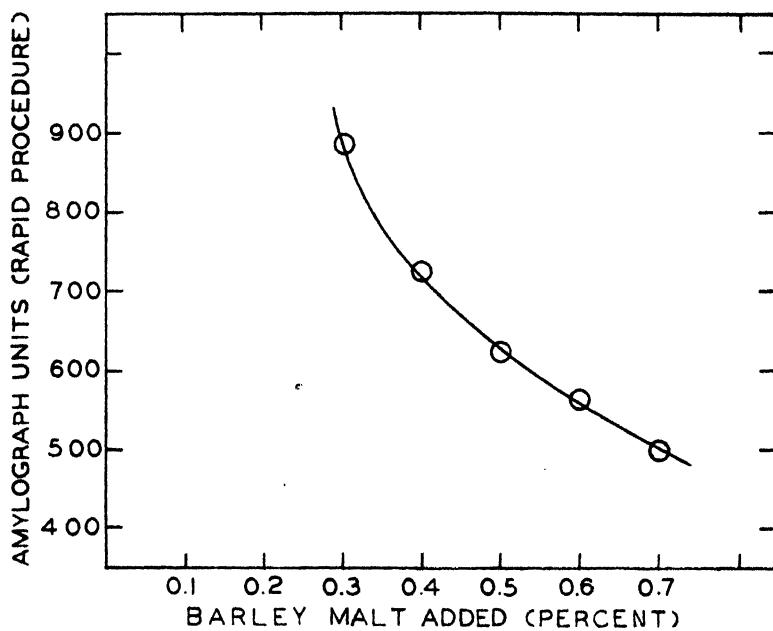


Fig. 1. Relation between maximum consistency of wheat flour pastes and quantity of barley malt added.

To check the correlation of the experimental procedure with amylograph values obtained by the standard procedure, 36 flour samples of varied types covering a wide range of alpha-amylase activity were analyzed by both procedures. The experimental values were plotted against the conventional amylograph values obtained by the procedure described by Selman and Sumner (4). The results are shown in Fig. 2. A straight line relationship exists between the two methods, and a positive correlation coefficient of 0.99 is shown. No attempt was made to hold the line voltage constant while obtaining these data. The variation was from 112 to 118 volts.

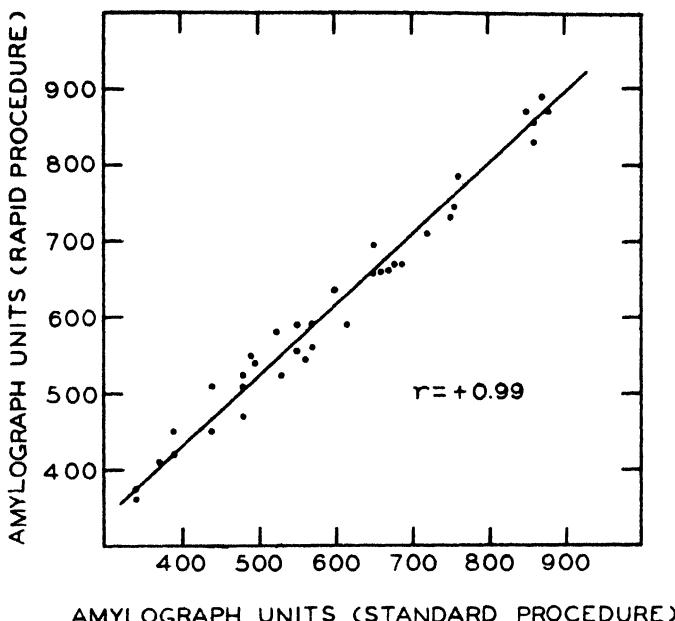


Fig. 2. Relation between maximum consistencies of flour pastes determined by the rapid and standard procedures.

Discussion

The initial temperature and the rate of temperature increase employed in this work were chosen arbitrarily, the object of the work being to determine whether malt activity could be satisfactorily evaluated by a method measuring only that effect of amylase which takes place after gelatinization has started. It is quite possible that the determination could be made at an initial temperature as high as 55°C., which would cut several minutes from the running time.

The rate of temperature increase is the factor which limits the sensitivity of the method, since a more rapid rate of heating would decrease the period of exposure of the gelatinized starch to the enzyme action, which would, in turn, decrease the consistency differential between the paste viscosity produced by a flour of low amylase content and that produced by a flour of high amylase content.

In routine mill diastatic control, this rate of temperature increase could be adjusted to suit the demands of the individual mill, since extreme sensitivity is of no advantage if the malt feeding equipment cannot accurately measure malt within narrow limits. It is obvious that by using the highest possible initial temperature, and by using the maximum rate of heating which will provide the desired degree

of sensitivity, a method of malt evaluation can be devised which will permit very rapid spot checks of the malt content of flour. Such a determination might be carried out in a sufficiently short period of time as to permit constant diastatic control of the flour in process, instead of providing a check on the flour already packaged.

To apply such a method, an instrument is needed which will meet the following requirements:

(1) It must indicate the maximum paste viscosity reached during the determination, but it is not necessary that a kymograph be used to record the entire course of consistency change.

(2) It must provide a reproducible rate of temperature increase, although the rate of temperature increment need not be constant, so long as this selected rate of temperature increase is reproducible from one determination to another. That is, immersion in a jacket or bath at constant temperature might be used instead of a system of uniform thermoregulation, if the jacket or bath were maintained at a temperature well above the gelatinization point of the flour suspension. A temperature of 95°C. or above is considered appropriate.

(3) It must be easily cleaned and durable in design.

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THE ACTION OF BETA-AMYLASE ON CORN AMYLOSE¹

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ABSTRACT

Corn amylose, having a degree of polymerization of 455 and an iodine binding power of 19.2%, was prepared in 20.8% yield from defatted corn starch by the butanol precipitation method of Schoch. After dissolving in alkali and adjustment of the pH, the hydrolysis of the amylose solution by beta-amylase was studied by removal of portions of the reaction mixture, analyzing for reducing sugars, and recovering the unconverted high polymeric material by alcoholic precipitation. Analytical results showed that, at least during the first half of the hydrolysis, substantially only unconverted amylose and sugars with a reducing value equal to maltose were present. Inspection of unconverted amylose residues showed, except in the final stages of the hydrolysis, an unexpectedly small decrease in viscosity and degree of polymerization values, a decrease in reducing value, and an increase in iodine complexing power, compared to the original amylose. These data show that when a hydrolysis of amylose by a limited addition of beta-amylase is interrupted, only sugar and unconverted residues which have very nearly the same average size as the original sample are obtained. These results may be interpreted by assuming that when the enzyme makes contact with an amylose molecule, this chain is hydrolyzed to maltose before the enzyme attacks another chain.

The action of beta-amylase on starch is known to be a successive splitting of maltose units, by hydrolysis, from linear structures. These may be either unbranched, alpha-glucopyranoside molecules or unbranched portions of more complexly constituted molecules. The end product in the case of unbranched amylose chains is maltose, and yields approximating 100% of theory have been reported (10, 7).

The purpose of this paper is to extend our knowledge of the mechanism of beta-amylase action.

Materials and Methods

Preparation of Corn A-Fraction (Corn Amylose). The linear polymer fraction of defatted corn starch was prepared according to the method of Schoch (13) using Pentasol in the primary separation and butanol to recrystallize the product from aqueous solution. The yield was 20.8%. Iodine absorption by the product, measured potentiometrically by a modification (8) of the method of Bates, French, and Rundle (2), was found to be 19.2%.

Preparation of Beta-Amylase. The enzyme was prepared from barley according to the procedure outlined by Kerr and Trubell (4) and further purified according to the method of Ohlsson (12). Free-

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dom from alpha-amylase was determined by using amylopectin, limit dextrin as substrate and noting the absence of new reducing groups formed, as measured by ferricyanide oxidation.

Enzyme Hydrolysis. Fifty grams of the corn amylose were dissolved at room temperature in 250 ml. of 2 N potassium hydroxide solution. The solution was diluted to 10 liters and adjusted to pH 5.8 with hydrochloric acid. This preparation was heated to 47°C., 25 ml. of a beta-amylase preparation were added, and the final volume adjusted to 12,500 ml.

Hydrolysis was allowed to proceed at 47°C. and aliquot portions were removed for testing and for the recovery of products at stated times. The per cent conversion to maltose at different time intervals was determined by reduction of ferricyanide according to the method used by Kerr, Trubell, and Severson (5).

Samples of the conversion liquors were taken at the times indicated, butanol added to the extent of 10%, by volume, the solution brought quickly to a boil, and partially cooled. Then another 10% by volume of butanol and a volume of methanol equal to the amount of water present were added. The solution was allowed to cool to room temperature by standing overnight. The precipitate was recovered by centrifuging and recrystallized by dissolving in 300 ml. of boiling water (115 ml. for sample C, Table I), saturating the solution with butanol,

TABLE I
HYDROLYSIS OF CORN AMYLOSE WITH BETA-AMYLASE

Sample taken for precipitation	Hydrolysis time	Hydrolysis to maltose	Monomolecular reaction rate constant	Unconverted, high polymer fraction		Characteristics of unconverted high polymer			
				Calculated	Found	Ferricyanide No.	Viscosity in ethylenediamine	Molecular extinction coefficient	Degree of polymerization
A	min.	%	$k_1 (min^{-1})$	g.	g.	[η]	$\times 10^{-3}$		
	0	0	0.0044	(50)	(50)	1.43	1.258	3.73	455
	30	10.3	0.0042	—	—	—	—	—	—
	60	19.7	0.0040	—	—	—	—	—	—
B	90	28.0	0.0035	6.6	6.2	1.33	1.162	4.13	330
	180	43.0	0.0030	—	—	—	—	—	—
C	260	52.6	0.0028	6.5	6.2	1.27	1.193	4.11	365
	1260	90.7	0.0006	2.1	0.9	6.30	0.240	3.06	120

and allowing the mixture to cool slowly overnight to room temperature. The recrystallized products were washed twice with 100-ml. portions of cold water saturated with butanol and then with four, 400-ml. portions of absolute methanol. The methanol was removed by plac-

ing the crystals in a vacuum desiccator over sulfuric acid at room temperature.

Measurement of Viscosity. The intrinsic viscosity of the various amylose products was determined in ethylenediamine using substantially the procedure described by Kerr (8).

Reducing Value. The ferricyanide reducing value of the products was determined in a manner analogous to the determination of percentage conversion of amylose. However, the results are expressed as milliliters of tenth normal sodium thiosulfate, equivalent to 1 g. of amylose product.

Molecular Extinction Coefficients. The molecular extinction coefficients of the iodine complexes were determined using a Coleman, Model 10S spectrophotometer. Exactly 0.1000 g. of amylose product was dissolved in 3.33 ml. of 2 *N* potassium hydroxide and transferred to a 2-liter flask with 1.5 liters of water. The pH was adjusted to 5.0 with a solution of hydrochloric acid and 20 ml. of a standard solution of iodine in potassium iodide was added. The latter contained 0.5 g. of iodine and 0.75 g. of potassium iodide per 100 ml. The volume of the mixed solution was made up to 2 liters. This solution was further diluted fivefold with water for spectrophotometric measurements over the wave band range of 550 to 660 $\text{m}\mu$. The per cent light transmission at the wave length which gave the minimum value was selected in each case for calculation of the molecular coefficient of extinction according to the equation:

$$k = - \frac{\log T}{dc}$$

wherein T is the fractional transmission

d is the cell depth of 1.3 cm

c is 0.047875 molar iodine concentration.

Acetylation. Acetates of the amylose products were prepared by a modification of the method of Mullen and Pacsu (11). The modification consisted of adding fused sodium acetate to the reaction mixture after distilling water from the aqueous pyridine solution and a primary reaction with acetic anhydride.

The reaction mixture was refluxed for two additional periods of one hour each after addition of the sodium acetate. By this modified procedure yields of acetates were obtained in excess of 90% of theory and which had acetyl contents between 44.0 and 44.8%.

Osmotic Pressure. Osmotic pressure measurements were made on the acetates of the amylose products in chloroform using a cell substantially as described by Fuoss and Mead (3). The cell was enclosed

in a glass-windowed cabinet which was maintained at 30°C. \pm 0.05 degree. Readings of liquid levels were made by the use of a Gaertner, model M908, cathetometer with an accuracy of \pm 0.05 mm. The dynamic method of measurement described by Fuoss and Mead was used. The calculated values were checked frequently with static values.

Membranes consisted of uncoated No. 450 cellophane which were treated by soaking successively in water (50°C.); 50% water—50% ethanol; 95% ethanol; 50% ethanol—50% amyl alcohol; 100% amyl alcohol; 75% amyl alcohol—25% chloroform; 50% amyl alcohol—50% chloroform; 25 per cent amyl alcohol—75% chloroform; and chloroform. Each soaking period consisted of from 6 to 24 hours.

The molecular weights, expressed as degree of polymerization (number average of glucose units), were calculated from the osmotic pressure of the acetates at zero concentration (by extrapolation) as follows:

$$DP_n = \frac{RT}{[\pi/c]_{c=0} \times m} = \frac{2.57 \times 10^6}{[\pi/c]_{c=0} \times 288}$$

where R is the gas constant

T is the absolute temperature

π is the osmotic pressure expressed as cm head of water

c is the concentration of the acetate in grams per 100 ml of chloroform

m is the molar weight of an anhydro, glucose triacetate group.

Results and Discussion

The results of the hydrolysis of corn A-fraction (amylose) by beta-amylase and of various tests performed on three intermediate samples recovered during the course of the hydrolysis are shown in Table I. The reaction rate of the hydrolysis does not correspond to a zero order reaction since equal weights of maltose are not formed in each successive unit of time. Neither does the monomolecular reaction equation, wherein the rate constant is a function of the amount of unconverted amylose, give constant values for K_1 although constant values have previously been reported (7) when the shorter amylose chains are hydrolyzed in dilute solution. For the whole amylose fraction of corn starch the rate constants decrease progressively as the hydrolysis continues. By extrapolation of these K_1 values to zero time, an initial K_1 value of 4.2×10^{-3} was estimated. This is less than half the value of 9.8×10^{-3} , found for the hydrolysis of an equal weight concentration of the shorter chain lengths such as are obtained by aqueous leaching of corn starch (6). The greater rate for shorter chains may be ex-

plained, in part at least, by the fact that there are a greater number of reactive chain ends, per unit weight, of the shorter than of the longer chain length sample.

The amounts of high polymeric material recovered from the conversion of corn A-fraction agree fairly well with calculations based on the assumption that at any time during the hydrolysis (at least up to 50% conversion) the only substances present are high polymers and sugars with a reducing value substantially equal to that of maltose.

The nature of the unconverted polymers is indicated by measurements of viscosity, reducing value, molecular extinction coefficient of the iodine complexes and osmotic pressure studies. Up to a point corresponding to 50% conversion of the amylose to maltose, the intrinsic viscosity of the unconverted material decreases very little. This result might be anticipated from the following considerations. The shortest amylose chains in the original sample would be expected to contribute little to its viscosity, since viscosity is a function of weight average molecular weight (9). It would also be expected from rate studies that a greater number of these shortest chains would be hydrolyzed per unit time than the longer, leaving an increasing proportion of longer chains as the hydrolysis time was extended.

However, if the reaction proceeded uniformly so that all molecules were progressively hydrolyzed, one would also expect that by the time half the weight of the amylose sample was converted, the longest chains would be materially reduced in chain length also. This is apparently not the case, particularly as in the present instance, when a limited amount of enzyme was used. Osmotic pressure measurements confirm this conclusion. Calculations of degree of polymerization from the osmotic pressures of the acetates in chloroform give a value of 455 glucopyranose units for the whole corn amylose sample and 365 units for the amylose which remains after 52.6% conversion to maltose. An examination of the reducing value of the amylose chains, which were recovered from the earlier stages of the hydrolysis, shows that there are very nearly the same number of reducing end groups per unit weight as in the original amylose. This is also fair evidence that the number average molecular weight of the recovered amylose is of the same order as that of the original material. Therefore it seems improbable that the action of beta-amylase is a gradual shortening of all chain lengths present because, if, at 50% conversion, the number of amylose chain residues were the same as the number of original amylose molecules, then the number average molecular weight at this point would be very nearly half of the original molecular weight.

Alternately, one may assume that the longer chains are preferentially hydrolyzed and that the range and distribution of molecular

sizes in the original amylose are such that when 50% conversion to maltose has been attained, the average chain length of the original sample is only slightly reduced as the result of the complete hydrolysis of a relatively few high molecular weight chains. However, if we assume that there are only unbranched chains in the amylose, then, in view of the work of Baldwin, Bear, and Rundle (1), our values for molecular extinction coefficients indicate that in the early stages of conversion the longer molecules of high iodine binding power have been preserved and that the sugar produced has been from the hydrolysis of the shorter chains of low iodine binding power. The value of 4.11×10^3 after 52.6% hydrolysis is significantly higher than 3.73×10^3 , the value for the original corn A-fraction.

The increased iodine staining power of the unconverted residue is not consistent with the assumption that its high average molecular weight is due to an accumulation of amylopectin impurities as the hydrolysis of linear chains progresses.

On the basis of present concepts the data given can be interpreted by concluding that once an active group on the enzyme makes contact with a starch chain, hydrolysis goes to completion before another starch chain is attached. At any time during the hydrolysis of linear chains the product is substantially all maltose; only a very small amount of intermediate products is present. Shorter chains are hydrolyzed at a greater rate, per unit weight, than longer and it seems probable also that the shorter chains are more readily contacted by the enzyme.³

The above conclusion is important when it is desired to modify the linear polymer characteristics of whole starch by amylase action. When beta-amylase is used for this purpose, or enzymes which contain this component, the linear polymer is modified in direct proportion to the production of sugar and in the modified starch product the proportionate weight of the linear component is eventually lost for applications where high polymeric materials are required.

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³ From the conclusion given for the mechanism of beta-amylase action it follows that by varying the ratio of the number of active enzyme groups to the number of amylose molecules present, one may obtain from the hydrolysis unconverted residues which have molecular weights less than, equal to, or even greater than the average for the original amylose. For example, if a large excess of enzyme were added so that there were more than one active enzyme group for each starch molecule, then one might expect that all amylose chains would be hydrolyzed simultaneously. On the other hand if only a very limited number of active enzyme groups were present, it would appear that only a very few of the short chain lengths would be hydrolyzed.

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ADSORPTION MEASUREMENTS ON FLOUR USING RADIOACTIVE ISOTOPES¹

J. W. T. SPINKS² and C. I. TOLLEFSON³

ABSTRACT

A convenient and rapid radioactive tracer method of determining adsorption of ruthenium ions by flour is described. The adsorption of Ru⁺⁺⁺ by four wheat flour mill streams increased with decreasing grade of flour, and it is possible that the method may be of value as a quick method for estimating baking quality on a small sample of flour.

During the last few years a number of studies have been made on the adsorption of different materials by flour (2). The amounts adsorbed are sometimes quite small, of the order of a few gammas per gram flour. While such amounts can ordinarily be measured without difficulty using a colorimetric procedure, the determination in the presence of flour is quite tedious. In contrast to this, the determination with the help of radioactive tracers is very simple and rapid. Furthermore, the extreme sensitivity of the radioactive tracer method enables one to work on a micro or semimicro scale, a fact which could be of considerable importance when making measurements, for example, on flour from a new variety of wheat. Since radioactive iso-

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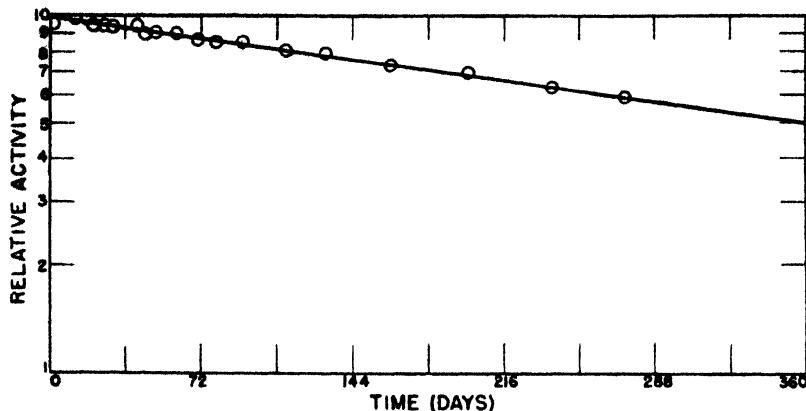
topes of practically all elements are now available in quantity, the radioactive tracer method is of general applicability and it was thought that a description of some experiments on the adsorption of ruthenium chloride by flour might be of some interest.

In any tracer experiment, a number of general factors are important. Among these are: (a) half life, (b) energy of the emitted radiations, (c) presence of interfering daughters, (d) self absorption, (e) counter standardization, and (f) statistical errors (3). In the present experiments, ruthenium (Ru^{106}), half life one year, was used. This half life is sufficiently long that no correction for the decay of Ru^{106} need be made in experiments completed within a day or so. Ru^{106} decays with emission of beta particles, energy 0.03 Mev, to give rhodium, half life 30 seconds. Rh^{106} decays with emission of beta particles, energy 3.9 Mev, to give stable palladium (Pd^{106}). The beta particles from Ru^{106} are not energetic enough to penetrate the window of the usual Geiger Mueller counter. However, any specimen of Ru^{106} which has been standing for a few minutes will be in equilibrium with its Rh^{106} daughter, whose beta particles are easily measurable. The radiations of the Rh^{106} daughter can, in fact, be used as a quantitative measure of the amount of Ru^{106} present.

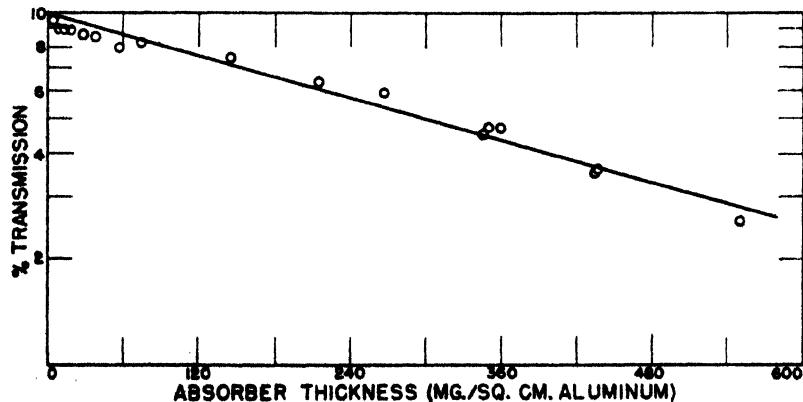
Materials and Methods

A sample of radioactive ruthenium was separated from possible impurities by a perchloric acid distillation (4). Fuming perchloric acid oxidizes ruthenium to the volatile tetroxide, RuO_4 , which distils off and is trapped in caustic soda solution. The octavalent ruthenium is readily reduced by alcohol to the trivalent state and the oxide, Ru_2O_3 (hydrated), precipitated. This may be centrifuged off, washed, and dissolved in a little hydrochloric acid. Half life and half thickness values indicated that the sample so purified was the Ru^{106} isotope. All samples were counted using a Geiger Mueller counter and a scale of 128 counting unit (7).

When determining the half life, the ruthenium sample was compared with a uranium oxide standard over a period of 9 months. The activity of the ruthenium was calculated on the basis that the activity of the uranium oxide standard remained constant over the period studied. This eliminated errors due to variations in counter performance. In all cases enough counts were recorded that the probable error should be less than $\pm 1\%$. All counts were corrected for background. The data for the half life of this ruthenium sample are plotted in Fig. 1. By extrapolation of the half life curve, it appears that the half life of the sample is about 360 days. The published value for the half life of Ru^{106} is one year (6).

Fig. 1. Half life of Ru¹⁰⁶.

In determining the half thickness, the net counting rate is determined with and without aluminum absorber screens interposed between the sample and the counter tube. The per cent transmission through a given screen is plotted against the absorber thickness expressed in mg. per sq. cm. From this curve, the half thickness value can be determined—i.e., the thickness of aluminum required to reduce the activity of the ruthenium sample to one-half its initial value. The data for this determination are plotted in Fig. 2.

Fig. 2. Half thickness of Ru¹⁰⁶.

The half thickness of Ru¹⁰⁶ as determined from Fig. 2 is 300 mg. per sq. cm. Al, and indicates that absorption of beta particles by the material itself (so called self-absorption) can be neglected.

During the course of the experimental work all radioactive residues were saved and were worked up on two occasions to recover the ruthe-

nium. The residues were combined and evaporated to dryness. Since considerable organic matter was present, and it was desired to remove the ruthenium by perchloric acid distillation, it was necessary to destroy the organic matter first. This was accomplished by means of a peroxide fusion in an iron crucible (5). The fusion cake was dissolved in water, partly neutralized with hydrochloric acid, and boiled to destroy excess peroxides. As some iron had dissolved from the crucible during the fusion, a precipitate of ferric hydroxide formed and carried down practically all the ruthenium. The precipitate was centrifuged off, washed, and subjected to a perchloric acid distillation. In the second recovery of ruthenium, the radioactive residues were evaporated to dryness and the ruthenium recovered by distillation with sulfuric acid and sodium bromate (1).

Procedure. One quarter gram samples of flour ⁴ were weighed into centrifuge tubes and treated with 5 ml. of solution containing radioactive ruthenium ion. The tubes were shaken by hand to disperse the flour and then placed on a "Gyrosolver" shaker for 10 minutes. The samples were next centrifuged and aliquots of the supernatant liquid taken for counting. These aliquots were dried on small watch glasses and counted. From the difference between the activities of the original and final solutions, the amount of ruthenium ion removed from solution could be calculated. In the absence of interfering effects, this was equal to the amount of ruthenium ion adsorbed by the flour. This was then converted to gammas of ruthenium ion adsorbed per gram of flour.

To determine the correction for self absorption, the residue on each watch glass was weighed. The weight varied between 5.1 and 8.8 mg., with an average value of 6.2 mg. The area of watch glass covered was about 5 sq. cm. Hence, the thickness of precipitate was in no case greater than 2 mg. per sq. cm. This would have no appreciable effect on the measured activity of these samples as ruthenium emits quite energetic radiation (maximum energy 3.9 Mev, half thickness 300 mg. per sq. cm.). Thus, for these experiments, it was not necessary to apply corrections for self absorption.

The method of calculating the amount of ruthenium ion adsorbed is quite simple. One quarter gram samples of flour were treated with 5 ml. of ruthenium chloride solution containing, say, 25 gammas Ru^{+++} . This corresponds to 100 gammas Ru^{+++} per gram flour. After shaking the sample and centrifuging, an aliquot of 1 ml. was withdrawn from the supernatant liquid. This aliquot was dried and had an activity of 566 counts per minute. A similar aliquot of the

⁴ Samples of freshly milled, unbleached, untreated flours were obtained from the Quaker Oats Company, Ltd., Saskatoon.

original solution had an activity of 813 counts per minute. Since the initial amount of ruthenium ion corresponded to 100 gammas Ru^{+++} per gram flour, the final amount of ruthenium ion in solution must correspond to $\frac{566}{813} \times 100 = 69.6$ gammas Ru^{+++} per gram flour. By difference, the amount of ruthenium ion adsorbed must have been 30.4 gammas Ru^{+++} per gram flour.

Factors Influencing Adsorption of Ruthenium

Effect of pH. In some preliminary experiments a marked effect of pH on the "adsorption" of ruthenium ion by flour was observed. Since it was thought that the effect might be due, at least in part, to precipitation of ruthenium, the effect of pH on the precipitation of Ru^{+++} was investigated.

Effect of pH on the Precipitation of Ru^{+++} . A series of ruthenium chloride solutions, with pH adjusted, were put through all the steps of the regular adsorption procedure, but with no flour added. The results are recorded in Table I.

TABLE I
EFFECT OF pH ON PRECIPITATION OF Ru^{+++} ($T = 20^\circ\text{C}.$)

pH	Activity left in solution, %
2.24	100.0
2.38	95.6
2.70	92.7
2.75	95.2
2.90	94.3
3.00	90.2
3.15	100.0
3.40	40.0
4.00	23.2
4.40	14.4
5.40	20.8
8.70	4.5

From Table I it is evident that ruthenium chloride solution is not stable above pH 3.0-3.2. Apparently, hydrolysis takes place at higher pH values and the hydrated oxides precipitate (1). In any case, it is necessary to carry out all the adsorption tests at as low a pH as is practicable in order to avoid this effect. As a result, it was decided to carry out all adsorption tests at pH 3.1.

Effect of Shaking Time. To determine the effect of time of shaking, samples of a "second break" flour were shaken with a ruthenium chloride solution for varying lengths of time on the Gyrosolver, centrifuged, and the activity of the supernatant determined in the usual manner. The results (see Table II) indicate that samples should be

shaken for at least 5 to 10 minutes to ensure complete dispersion of the flour in the ruthenium chloride solution. It appears that fairly reproducible results can be obtained by this method—the probable error for this experiment was $\pm 2\%$.

TABLE II
EFFECT OF TIME OF SHAKING (T = 20°C.)

Sample	Shaking time, minutes	Ru ⁺⁺⁺ added, gamma	Activity lost, %	Ru ⁺⁺⁺ adsorbed, gamma
1	0.5	25	27.6	6.9
2	0.5	25	29.6	7.4
3	5	25	39.6	9.9
4	5	25	39.2	9.8
5	10	25	41.2	10.3
6	10	25	39.6	9.9
7	15	25	39.2	9.8
8	15	25	40.0	10.0

Effect of Concentration of Ru⁺⁺⁺ and Grade of Flour. In order to investigate the adsorption of ruthenium ion by flour in somewhat greater detail, six samples of each of four mill streams were treated with different concentrations of ruthenium chloride solutions. These samples were shaken on the Gyrosolver for 15 minutes, centrifuged, and the activity of the supernatant liquid determined in the usual manner. The results are recorded in Table III.

TABLE III
RESIDUAL AMOUNTS OF RU⁺⁺⁺ IN SOLUTIONS AFTER TREATMENT WITH FLOURS¹
(GAMMAS RU⁺⁺⁺ PER GRAM FLOUR)

Initial amount of Ru (gammas Ru ⁺⁺⁺ / gram flour)	First middlings	Second break	Third low grade	Third tailings
18.75	10.9	8.0	7.4	7.2
37.50	25.0	21.1	17.0	14.2
75.00	53.3	50.5	45.4	36.9
150.00	114.3	115.3	111.1	104.6

¹ Ash contents: first middlings 0.36%, third low grade 0.83%, second break 0.46%, third tailings 0.62%.

When the amount of ruthenium ion adsorbed is plotted against the amount of ruthenium ion remaining in solution, typical adsorption curves are obtained.

The results in Table III also indicate that the amount of ruthenium ion adsorbed increases with decreasing flour grade. For more extensive data see Tollefson (8). This parallels the requirement of im-

prover for optimum loaf volume, and indicates the possibility of correlating flour grade with adsorption of ruthenium ion.

It is known that certain flours improve in baking quality on aging and, consequently, adsorption tests were made on a number of mill streams over a period of several months. Typical results are recorded for one flour in Table IV.

TABLE IV

RESIDUAL AMOUNTS OF RUTHENIUM IN SOLUTION AFTER TREATMENT WITH FIRST MIDDLES AT VARIOUS DATES (GAMMAS Ru^{+++} PER GRAM FLOUR)

Initial amount of Ru^{+++} , (gammas Ru^{+++} /gram flour)	Nov. 20/46	Nov. 30/46	Dec. 18/46	Jan. 5/47	Feb. 13/47	Mar. 22/47
18.75	10.9	11.3	10.8	11.7	11.4	10.9
37.50	25.0	24.9	23.9	24.8	24.0	24.2
75.00	53.3	54.7	53.3	54.4	53.7	53.6
150.00	114.3	116.8	114.3	115.5	114.3	116.7

During the period studied there was no appreciable change in the adsorption of ruthenium ion by the different flour streams. However, during the same period, there was no appreciable change in loaf volume so that no particular conclusions can be drawn.

Acknowledgment

The authors are grateful to the National Research Council of Canada for a grant in aid of research.

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WATER SORPTION BY CORN STARCH AS INFLUENCED BY PREPARATORY PROCEDURES AND STORAGE TIME¹

N. N. HELLMAN and E. H. MELVIN

ABSTRACT

Measurements were made of the amount of water sorbed by 10 corn starches in equilibrium with atmospheres of different relative humidities ranging from 12 to 93%. The 10 starches differed among themselves with respect to origin, concentration of sulfur dioxide in the steep water at the time of extraction, drying, and time in storage. Despite these differences in preparation, all 10 starches showed essentially the same moisture sorption. It was concluded that data secured in studies of the moisture-sorptive properties of a single corn starch can generally be applied to normally prepared unmodified corn starches.

The amount of water which corn starch will sorb in equilibrium with an atmosphere of given moisture content has been shown to be affected by intensive drying and the various treatments used commercially to prepare modified starches (2, 3). The moisture-sorptive capacity will also vary, depending upon (a) the difference between the initial and final moisture content of the starch and (b) whether moisture is being lost or gained by the starch in the approach to equilibrium. Changes in water-sorptive capacity of the latter type are analogous to those shown by many other colloidal materials, and the phenomenon is known as hysteresis. The effects of hysteresis are reversible and are thus distinguished from the permanent changes in sorptive capacity which are induced by the first-mentioned treatments.

Permanent differences in water-sorptive properties might conceivably be produced also by variations encountered normally in the preparation of starch, such as difference in origin and condition of the corn, extraction and drying procedure, and time of storage. A survey is here reported of the moisture-sorptive properties of a collection of corn starches which differed among themselves with respect to the factors just mentioned. This survey was preliminary to a detailed study of moisture sorption isotherms of corn starch and was made to determine if data obtained in such a study would be generally applicable to unmodified corn starches.

Materials and Methods

Ten corn starch samples were selected which represent different preparative methods and periods of storage. The pertinent properties of the corn starch samples are given in Table I. Approximately 2-g.

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Contribution from the Northern Regional Research Laboratory, Peoria, Illinois, one of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

TABLE I
PREPARATORY HISTORY OF CORN STARCH SAMPLES

Sample No.	Processor	Type	Concentration of SO_2 in steep water	Method of drying	Temp. of drying, °F.	Time of drying	Time in storage (months)
L-1	Laboratory	Prepared from Iowa hybrid 939 corn (1942 crop)	0.20% ¹	Forced draft oven	105	7 hrs.	33
L-2	Laboratory	Prepared from Iowa hybrid 939 corn (1942 crop)	0.30% ²	Forced draft oven	105	7 hrs.	39
L-3	Laboratory	Prepared from Iowa hybrid 939 corn (1942 crop)	0.40% ²	Forced draft oven	105	7 hrs.	37
L-4	Laboratory	Prepared from Iowa hybrid 939 corn (1942 crop)	Distilled water used	Forced draft oven	105	3 hrs.	13
PP-1	Pilot Plant	Prepared from Iowa hybrid 939 corn (1942 crop)	Distilled water used	Forced draft oven	110	26 hrs.	17
C-1 ²	Company A	Prepared from soft corn Pearl	Normal 0.02-0.08	Proctor Schwartz Kiln	20 hrs.	56	1
C-2	Company A	Powdered	Max. 0.18	Proctor Schwartz	25 min.	55	
C-3	Company B	Pearl	0.04-0.20	Proctor Schwartz	1 hr.	56	
C-4	Company C	Pearl	0.04-0.20	Tunnel kiln	18 hrs.	56	
C-5	Company C	Pearl					

¹ For the laboratory preparations, the SO_2 concentration is that of the steep when started. No further SO_2 is added, and the water is not changed during the process.

² Scott viscosity only 15, whereas that for normal corn starch is 100 or over.

samples of the various starches at the moisture content at which they were normally stored (10-13%) were placed in aluminum moisture dishes. The samples were then placed in a vacuum desiccator over a saturated salt solution selected to give the appropriate vapor pressure of water. The solutions used are given in Table II and were selected

TABLE II
SALT SOLUTIONS USED FOR CONSTANT RELATIVE HUMIDITY

Salt	Relative humidity	Reference
KNO ₃	93.5	(4)
KCl	85.0	(4)
NaNO ₃	74.5	(4)
NaBr	56.4	(1)
K ₂ CO ₃	43.0	(4)
KC ₂ H ₃ O ₂	20.0	(4)
LiCl	12.2	(1)

from the *International Critical Tables* (4) and the *Physikalisch-chemische Tabellen* (1) tabulations. To hasten equilibrium, the desiccators were evacuated with a two-stage, rotary, oil, vacuum pump and, to ensure complete removal of air, were pumped for 1 hour after the pressure had been reduced to the pressure of the water in the salt solution. The desiccators were then placed in a constant-temperature room, controlled at 25 \pm 1°C., and allowed to stand 2 to 4 weeks, a period shown by other trials to be ample for equilibration.

At the conclusion of the period of equilibration the pressures of the atmospheres within the desiccators were measured with an ordinary closed-end, mercury, U-tube manometer to assure that the pressure of water was that anticipated from the literature data. Dry air was then admitted, the desiccator opened, and the moisture content of the starch samples determined. Moisture contents were determined by heating in a vacuum oven at 105°C. for 24 hours. Tests of the reproducibility of results by use of this technique showed that within the same desiccator the per cent moisture content of triplicate samples agreed to within 0.3. The vapor pressures of water in the desiccators probably varied slightly for a given salt solution as a result of slight under or super saturation during the course of equilibration inasmuch as the rate of salt solution or precipitation probably could not follow the rate of temperature drift. This gives rise to an uncertainty in the relative humidities designated in Table III, which was reflected in the manometer readings and in a variation in the per cent of water by as much as 0.6 encountered in the moisture content of a standard starch between duplicate desiccators employing the same salt.

TABLE III

WATER SORPTION OF CORN STARCH SAMPLES AT VARIOUS HUMIDITIES AT 25°C.

Sample	Per cent water sorbed in equilibrium with air of designated relative humidity						
	93%	85%	75%	56%	43%	20%	12%
L-1	26.5	22.1	19.4	16.7	13.9	10.6	7.7
L-2	26.1	22.1	19.5	16.7	—	10.6	7.6
L-3	26.3	22.1	19.6	16.3	14.2	10.6	—
L-4	27.0	22.3	19.7	16.3	14.2	10.6	7.6
PP-1	26.4	21.9	19.2	16.0	13.9	10.5	7.6
C-1	25.7	22.0	18.9	15.8	13.5	—	7.2
C-2	26.6	21.8	19.1	15.5	13.2	10.3	7.5
C-3	26.2	22.2	19.0	15.7	13.4	10.4	7.6
C-4	25.6	21.9	18.9	15.7	13.4	10.4	7.7
C-5	26.0	22.1	19.0	16.0	13.8	10.6	7.6
Average	26.2	22.0	19.2	16.0	13.7	10.4	7.6
PP-1-wet	26.5	22.7	20.0	—	14.2	10.3	7.4
C-1-wet	26.9	22.9	20.0	16.6	14.1	10.3	7.4.

Results and Discussion

The uniformity of moisture sorption by the various corn starches is particularly striking. The amounts of water sorbed by the starches at the seven humidities employed are shown in Table III. The concentration of sulfur dioxide in the steep water is shown to have little effect on moisture sorption by the first four starches tabulated. Sample SD-144, which was extracted from soft corn, appears, with respect to its moisture-sorptive properties, to have been relatively unaffected by its origin. The starches from laboratory extractions exemplified by samples L-1, L-2, L-3, and L-4 show little difference with respect to the water-sorptive properties as compared to commercial preparations C-1, C-2, C-3, C-4, and C-5. These laboratory and commercial preparations differed from each other in time of storage as well as in methods of drying. Although rigorous drying and modification treatments have been shown to affect moisture-sorptive properties (2, 3), it appears that within limits of normal corn starch preparatory practice, the variables of origin, sulfur dioxide concentration in the steep liquor, drying practice, and time of storage are of little significance. For moisture sorption studies, a single representative corn starch can be studied with confidence as to the generality of the results as applied to other unmodified corn starches. This conclusion cannot be applied to starches from other plant sources without further investigation, since it has been shown for potato starch that a preparative treatment involving combined heat and moisture would produce changes (2).

Within the general conclusion that the samples showed uniform sorption, the possibility exists that variations in preparative history may cause minor variations in water sorption. Such variations must be so small, however, that they can be obscured by the nonpermanent changes in moisture sorption shown in hysteresis which are of the same order of magnitude as the differences in sorptive capacities shown by the various starches studied. The effect of hysteresis is shown in samples of pilot-plant corn starch (PP-1-wet) and the freshest available (C-1-wet) commercial corn starch which were wet with sufficient water to cover completely the starch before equilibrating with moist air.

The viscosities of pastes from these materials lay over a relatively wide range.² The uniformity of the moisture-sorptive properties and the nonuniformity of their paste viscosities clearly indicate that hydration measured in terms of moisture sorption of whole granules at room temperature is not of primary importance in controlling the paste viscosity. If one wishes to consider that paste viscosity is controlled by hydration, then it must be hypothesized that this is effected through new sites of hydration which arise in the process of gelatinization.

Acknowledgment

The authors wish to thank Dr. M. M. MacMasters of this laboratory for providing the samples used in this investigation and her helpful discussion during the progress of this work. The interest of Dr. R. T. Milner is gratefully acknowledged.

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²Private communication from Dr. M. M. MacMasters, Northern Regional Research Laboratory, 1947.

BOOK REVIEWS

Volumetric Analysis. Volume II, Titration Methods. By I. M. Kolthoff and V. A. Stenger. 374 pp. Interscience Publishers, New York, N. Y. 1947. Price \$6.00.

This book is an extensively revised and expanded portion of the well-known 1928 volume by Kolthoff and Furman. The present volume, *Titration Methods*, is a companion to volume I of the series, *Theoretical Fundamentals*, published in 1942. The book includes the general sections of acid-base reactions and of quantitative precipitation and complex formation reactions. The revised portion of the latter half of the earlier text on oxidation reduction methods is scheduled for publication as volume III in 1948.

The presentation has the same objectives and follows the same pattern as in the earlier volume. The authors give critical, detailed presentations of a somewhat arbitrary selection of methods. In the discussion of procedures the value of the wealth of experience of the authors is apparent. Specific recommendations are sometimes given, but on the whole the authors properly prefer to present different methods or variations together with ample literature references, and to thus allow the analyst to choose the procedure he deems best. In the presentation of methods, emphasis has been on measurement of inorganic or organic constituents of prepared or relatively pure samples. There is little information on the applications of the methods to analysis of biological materials.

The material presented in the original volume has been almost wholly retained; the expansion is due to revision and inclusion of new material. Enlargement of the section on nitrogen determination and presentation of more methods for various organic substances and for fluorine may be of interest to the biochemist. Later procedures are frequently given; the inclusion of a modification of Conway's micro-diffusion method for microdetermination of ammonia is a good example.

The methods are grouped according to the principle involved rather than the substance being determined, the purpose being to emphasize the principle of the determination. The value of this classification is weakened by the difficulty of making unequivocal decisions and by the frequent necessity of classifying a method on basis of only a small part of the procedure. In addition, it is necessary for the user to consult several portions of the volume for the various methods that may be presented for determination of one substance. Cross references in this regard are not always adequate.

The presentations are confined almost entirely to volumetric methods. To many this may be an inconvenient limitation, especially since reference to or mention of other procedures, which may be of equal or greater value than the volumetric method, is not made.

In their grouping of material and presentations the authors use the older established concepts of acids and bases rather than newer definitions based on Brönsted's concepts. Some other omissions of more recent views are apparent. For example, in presenting the formol titration of amino acids, formaldehyde is represented as combining with the amino group to form a Schiff base, in neglect of the considerable evidence against this mechanism. In this same regard the reviewer is entirely unaware of the necessity of the authors to note ". . . an interesting fact commonly ignored in physiological chemistry. The effect of alcohol on amino acids is not directly comparable with that of formaldehyde."

The clarity of presentation has been improved considerably over the earlier volume by use of better sentence structure and by more modern page make-up. Ambiguities and contradictions are rare, as are typographical errors. Some readers may object to the frequent use of relatively small type.

The preceding statements serve only to point out some limitations in an excellent book as evaluated for usefulness to a biochemist. The value and rarity of critical, detailed presentations in methodology, which made the earlier edition of this book a success, assure wide welcome and usage of the present volume.

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The Chemistry of the Carbon Compounds. Volume IV. The Heterocyclic Compounds and Organic Free Radicals. By Victor von Richter. Edited by Richard Anschütz and translated from Volume III and Volume II, part 2, of the 12th German edition by M. F. Darken and A. J. Mee. xv + 498 pp. Elsevier Publishing Co., Inc., New York, N. Y. 1947. Price \$12.00.

Richter-Anschütz has long been one of the classical reference texts of organic chemists and it is with regret that one finds that the present translation of the Heterocyclic Compounds and Organic Free Radical sections of the German edition has not been brought up to date. Unfortunately, this volume, like the previously published Volume III of the English edition, has been a victim of the war and the publishers have chosen to print a literal translation of the German edition rather than to delay or discontinue the publication of this series. It is felt that this was an unfortunate decision since as a result the Heterocyclic section covers the literature only through 1931 and the Organic Free Radical section through 1935. Thus the book loses some of its value as a reference text. One valuable feature of the present translation is that wherever possible the references are given to the original journals and not to *Chemisches Zentralblatt*, and names of authors have been added.

The pattern of the book is similar to previous volumes of this series in that chemical reactions, preparations, and properties are given fairly exhaustive treatment, while few theoretical discussions are given. Division is in two major parts, I. *The Heterocyclic Compounds* by F. Reindel (383 pp.) and II. *Organic Free Radicals* by Ludwig Anschütz (69 pp.). The division under part I is based on the number of atoms in the ring with further subdivision according to the number of hetero atoms in the ring. The part on Free Radicals is divided into a short General and a Special Part with the latter being further divided into sections based on the types of free radicals involved, i.e., those of carbon, nitrogen, oxygen, sulfur, chromium, and other elements of the third, fourth, and fifth vertical series of the periodic system.

The book is satisfactorily printed and bound, and although it does not have an author index it has an adequate subject index of 49 pages. The price of \$12.00 is somewhat high for the private library when one considers its potential value as an up-to-date reference.

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Technique de la Désinsectisation. By A. L. Lepigre. 270 pp., 31 figures. A. Joyeux, Alger. 1947.

This book is devoted largely to a discussion of the use of fumigants for the control of household and stored product insects. Other phases of fumigation considered are soil fumigation, fruit-tree fumigation and the possibility of using poison gas to combat field infestations of grasshoppers. Information is also included relative to the use of contact and residual sprays in houses, warehouses, and mills and to the use of chemically inert and chemically active dusts for the protection of stored grain.

The subject matter of the various chapters is indicated by the following titles: Chapter 1. Importance of insects to modern man; 2. Principal enemies; 3. Resistance of insects to control methods; 4. Insect protection—control methods; 5. Fumigation of packaged commodities; 6. Insecticidal agents—characteristics—dosages; 7. Physical properties of gases affecting fumigations; 8. Influence of temperature and humidity; 9. Influence of local conditions and stacking of merchandise on the efficacy of fumigants; 10. Materials—Methods—Precautions; 11. List of materials required for mobile fumigator; 12. Detection of HCN and methyl bromide; 13. Gas intoxications—first aid; and 14. Legislation regarding fumigations.

The information contained in the book is of a highly practical nature and is based on the practical experience of the author combined with a careful review of the literature on the subject. The bibliography contains 513 references.

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SUGGESTIONS TO AUTHORS

General. From January 1, 1948, an abstract will be printed at the beginning of each paper instead of a summary at the end, references will be numbered to provide the option of citing by number only, and date of receipt, author's connections, etc., will be shown in footnotes. Except on these points, authors will find the last volume of *Cereal Chemistry* a useful guide to acceptable arrangements and styling of papers. "On Writing Scientific Papers for Cereal Chemistry" (*Trans. Am. Assoc. Cereal Chem.*, 6: 1-22, 1948) amplifies the following notes.

Authors should submit two copies of the manuscript, typed double spaced with wide margins on 8½ by 11 inch white paper, and all original drawings or photographs for figures. If possible, one set of photographs of figures should also be submitted. Originals can then be held to prevent damage, and the photographs can be sent to reviewers.

Titles and Footnotes. Titles should be specific, but should be kept short by deleting unnecessary words. The title footnote shows "Manuscript received . . ." and the name and address of the author's institution. Author footnotes, showing position and connections, are desirable although not obligatory.

Abstract. A concise abstract of about 200 words follows title and authors: It should state the principal results and conclusions, and should contain, largely by inference, adequate information on the scope and design of the investigation.

Literature. In general, only recent papers need be listed, and these can often be cited more advantageously throughout the text than in the introduction. Long introductory reviews should be avoided, especially when a recent review in another paper or in a monograph can be cited instead.

References are arranged and numbered in alphabetical order of authors' names and show author, title, journal, volume, first and last pages, and year. The list is given at the end of the paper. Reference numbers must invariably be cited in the text, but authors' names and year may be cited also. Abbreviations for the names of journals follow the list given in *Chemical Abstracts* 40: I-CCIX, 1946.

Organization. The standard organization involves main sections for abstract, introduction, materials, methods, results, discussion, acknowledgments, and literature cited. Alternately, a group of related studies, each made with different materials or methods, may require a separate section for each study, with subsections for materials and methods, and for results, under each section. Center headings are used for main sections and italicized run-in headings for subsections, and headings should be restricted to these two types only.

Tables. Data should be arranged to facilitate the comparisons readers must make. Tables should be kept small by breaking up large ones if this is feasible. Only about eight columns of tabular matter can be printed across the page. Authors should omit all unessential data such as laboratory numbers, columns of data that show no significant variation, and any data not discussed in the text. A text reference can frequently be substituted for columns containing only a few data. The number of significant figures should be minimized. Box and side headings should be kept short by abbreviating freely; unorthodox abbreviations may be explained in footnotes, but unnecessary footnotes should be avoided. Leader tables without a number, main heading, or ruled lines are often useful for small groups of data.

Tables should be typed on separate pages at the end of the manuscript, and their position should be indicated to the printer by typing "(TABLE I)" in the appropriate place between lines of the text. (Figures are treated in the same way.)

Figures. If possible, all line drawings should be made by a competent draftsman. Traditional layouts should be followed: the horizontal axis should be used for

the independent variable; curves should be drawn heaviest, axes or frame intermediate, and the grid lines lightest; and experimental points should be shown. Labels are preferable to legends. Authors should avoid identification in cut-lines to be printed below the figure, especially if symbols are used that cannot readily be set in type.

All drawings should be made about two to three times eventual reduced size with India ink on white paper, tracing linen, or blue-lined graph paper; with any other color, the unsightly mass of small grid lines is reproduced in the cut. Lettering should be done with a guide using India ink; and letters should be $\frac{1}{8}$ to $\frac{1}{16}$ inch high after reduction.

For difficult photographs, a professional should be hired or aid obtained from a good amateur. The subject should be lighted to show details. A bright print with considerable contrast reproduces best, and all prints should be made on glossy paper.

All original figures should be submitted with one set of photographic reproductions for reviewers, and each item should be identified by lightly writing number, author, and title on the back. Cut-lines (legends) should be typed on a separate sheet at the end of the manuscript. "Preparation of Illustrations and Tables" (*Trans. Am. Assoc. Cereal Chem.* 3: 69-104, 1945) amplifies these notes.

Text. Clarity and conciseness are the prime essentials of a good scientific style. Proper grouping of related information and thoughts within paragraphs, selection of logical sequences for paragraphs and for sentences within paragraphs, and a skillful use of headings and topic sentences are the greatest aids to clarity. Clear phrasing is simplified by writing short sentences, using direct statements and active verbs, and preferring the concrete to the abstract, the specific to the general, and the definite to the vague. Trite circumlocutions and useless modifiers are the main causes of verbosity; they should be removed by repeated editing of drafts.

Editorial Style. A.A.C.C. publications are edited in accordance with *A Manual of Style*, University of Chicago Press, and *Webster's Dictionary*. A few points which authors often treat wrongly are listed below:

Use names, not formulas, for text references to chemical compounds. Use plural verbs with quantities (6.9 g. were). Figures are used before unit abbreviations (3 ml.), and % rather than "per cent" is used following figures. All units are abbreviated and followed by periods, except units of time, which are spelled out. Repeat the degree sign (5°-10° C.). Place 0 before the decimal point for correlation coefficients ($r = 0.95$). Use * to mark statistics that exceed the 5% level and ** for those that exceed the 1% level; footnotes explaining this convention are no longer required. Type fractions on one line if possible, e.g., $A/(B + C)$. Use lower case for farinograph, mixogram, etc., unless used with a proper name, i.e., Brabender Farinograph. When in doubt about a point that occurs frequently, consult the Style Manual or the Dictionary.

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METHODS FOR DETERMINING FLOUR PARTICLE SIZE DISTRIBUTION¹

FRANK W. WICHSER and J. A. SHELLENBERGER²

ABSTRACT

The determination of the particle size distribution of wheat flour by sieving, air flotation, and sedimentation procedures is discussed. Data produced by a sieving procedure using Tyler wire screens and the Ro-Tap-shaker are consistently accurate, applicable to any particle size range above 37μ , and simple to run. Flour separation by air using the Roller Air Analyzer produces particle size distribution data below the size of 80μ . The air procedure is more rapid than the other methods. A sedimentation method using the Andreasen pipette only produced accurate data below the size of 50μ .

The importance of a satisfactory method for determining the particle size distribution of flour was recognized long ago. Flour particles vary in size and exhibit marked differences in chemical and physical characteristics. Baking characteristics are partially influenced by the granularity of a flour.

Numerous investigations employing various methods have been conducted for determining flour particle size. While certain of these methods give reproducible results under the conditions of the test, they do not indicate the true particle size distribution. In developing reliable and accurate procedures it is necessary to compare data obtained by different methods with the same flour under equivalent standard conditions.

The method used extensively by previous investigators was one employing silk bolting cloths in a stack arrangement. The limitation of mesh fineness of the silk cloth and inaccuracies in the shape and size of the aperture openings presented serious drawbacks. Other limitations resulted from flour agglomerates, inadequate shaking devices, and the stack sieve arrangement.

A classification of the many possible methods for determining

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particle size distribution based on that of Scheyer and Work (10) is given below. Some of these methods may not be applicable to flour and others have been omitted because of insufficient data to justify a comparison with commonly used methods.

CLASSIFICATION OF METHODS FOR DETERMINING SIZE AND DISTRIBUTION OF PARTICLES

- I. Sieve Analysis
- II. Microscopic Analysis
 - A. Conventional microscopic methods
 - B. Ultramicroscopic methods
- III. Sedimentation Analysis
 - A. Increment methods:
 1. Pipette method
 2. Hydrometer method
 3. Pressure method
 4. Photographic method
 - B. Cumulative methods:
 1. Balance methods
 2. Pressure methods
- IV. Centrifugal Analysis
 - A. Ordinary centrifuge methods
 - B. Supercentrifuge methods
 - C. Ultracentrifuge methods
- V. Elutriation Analysis
 - A. Air elutriation
 - B. Liquid elutriation
- VI. Turbidimetric Analysis
 - A. Gross methods
 - B. Size distribution methods
- VII. Miscellaneous Methods
 - A. Permeability methods
 - B. Adsorption methods

The purpose of this investigation was to develop methods of wire sieve analysis, air elutriation, and sedimentation analysis, and to compare the results to determine the validity of each method for particle size distribution analysis of flour.

Materials and Methods

Commercially milled hard red spring, straight grade wheat flour was used, employing the one-half height W. S. Tyler standard screen scale sieves Nos. 100, 115, 150, 170, 200, 250, 270, 325, and 400 with the Tyler Ro-Tap shaker, the Roller Particle Size Air Analyzer, and the Andreasen pipette sedimentation column.

Sieve Analysis. The sieve analysis is a simple, accurate method of fractionating flour, but it is of little value unless made with a sieve having uniform square mesh openings. As silk bolting cloth has neither a uniform square mesh nor a sufficiently fine mesh to give accurate results, this precludes its use for flour granulation studies.

The Tyler Standard Screen Scale Testing Sieves, with aperture openings in the fixed ratio of the square, or fourth, root of two (1.414 and 1.189 respectively), fulfill the requirements for an accurate mesh sieve and are widely used for accurate particle size analysis. The sieves, used with the Ro-Tap shaker, produce dependable results if a

TABLE I
SIEVING DATA OBTAINED USING TYLER WIRE SCREENS AND RO-TAP SHAKER

Sieve mesh	Sifting time, min.	Material passing the sieve		Sieve mesh	Sifting time, min.	Material passing the sieve	
		I	II			I	II
400	1	7.6	5.4	200	1	34.2	17.6
	2	12.4	9.6		2	47.0	29.2
	3	15.6	12.4		3	52.4	36.4
	4	17.6	14.8		4	55.0	42.8
	5	19.0	16.4		5	56.8	45.2
	6	20.0 ¹	17.6		6	58.4 ¹	47.2
	7	20.8	18.4 ¹		7	59.2	48.8
	8	21.4	19.2		8	60.2	50.8
325	1	5.6	5.2	170	10	61.4	53.6
	2	9.8	9.8		11		54.8
	3	13.8	13.8		12		56.4 ¹
	4	17.2	17.2		13		57.2
	5	19.6	20.0				
	6	22.0	22.4		1	42.8	40.8
	7	23.8	24.0		2	57.4	54.4
	8	25.0	25.6		3	63.4	60.4
	9	26.2	26.4		4	66.8	63.6
	10	27.0 ¹	27.6 ¹		5	69.0	66.0
	11	27.8	28.4		6	70.4	67.8
270	1	17.0	14.6	150	7	71.6	69.2
	2	23.6	23.8		8	72.8 ¹	70.4
	3	27.0	28.6		9	73.6	71.6
	4	29.4	31.0		10	74.4	72.6 ¹
	5	30.8	32.8		11		73.4
	6	32.0	34.8		1	68.8	65.8
	7	33.2 ¹	35.2 ¹		2	77.8	75.4
	8	34.0	36.0		3	81.2	78.8
250	1	19.4	12.2	115	4	83.0	80.8
	2	28.8	19.4		5	84.4	82.6
	3	34.4	24.4		6	85.6 ¹	83.6
	4	37.8	29.8		7	86.4	84.6 ¹
	5	40.4	33.4		8		85.2
	6	42.0	36.2		1	87.0	84.2
	7	43.2	38.6		2	92.2	91.2
	8	44.4	40.4		3	93.8	93.8
	9	45.6	41.8	100	4	95.0 ¹	94.8
	10	46.6 ¹	43.8		5	95.6	95.8 ¹
	11	47.4	44.2		6		96.4
	12	48.0	45.2		1	95.4	94.0
	13		46.2 ¹		2	98.0 ¹	97.4
	14		47.0		3	98.6	98.4 ¹

¹ Optimal per cent of material passing sieve.

satisfactory procedure, such as that described by Wichser, Shellenberger, and Pence (13), is employed.

Data obtained using the above-named procedure for fractionating the hard red spring flour are presented in Table I. A footnote 1 indicates the point where the optimal per cent of material of each size has passed through the sieve. Additional material removed by continued sieving was probably the result of attrition and this amounts to approximately 0.8 per cent or less per minute. The number of minutes required to reach the end point varies with sieving conditions;

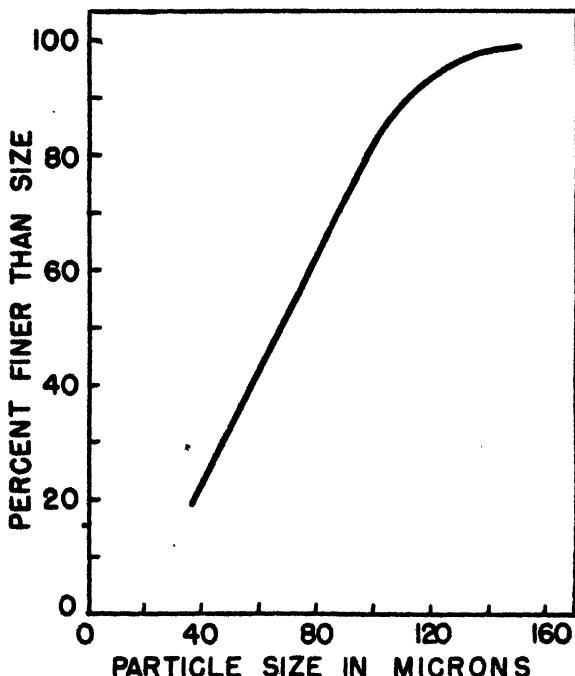


Fig. 1. Particle size distribution curve for wheat flour obtained by sieving.

however, the optimal per cent of material passing the sieve remains approximately the same for duplicate runs. A summation of the end point for all of the sieves establishes the particle size distribution curve illustrated in Fig. 1.

There are many advantages in the graphic method of showing the data obtained in screen analysis. Of the several methods of plotting these curves, the cumulative direct plot is most valuable and generally used. It is easy with a cumulative direct plot to find percentages that will pass, or be retained by, openings other than those used in the sieve test, and, conversely, to find the opening required to pass or re-

tain a designated percentage. Fig. 2 shows the data from Table 1 plotted by the cumulative direct plot method in the ratio of the fourth root of two (12). Particle size distribution curves for a straight grade winter wheat flour, and a straight grade soft wheat flour are also shown.

Air Elutriation. The Roller Particle Size Air Analyzer, described by Wichaer, Shellenberger, and Pence (13), is a laboratory instrument used as standard equipment in other dry powdered industries (8) for determining particle size distribution. The instrument was designed to apply Stokes' Law to the separation of a powder mass of uniform

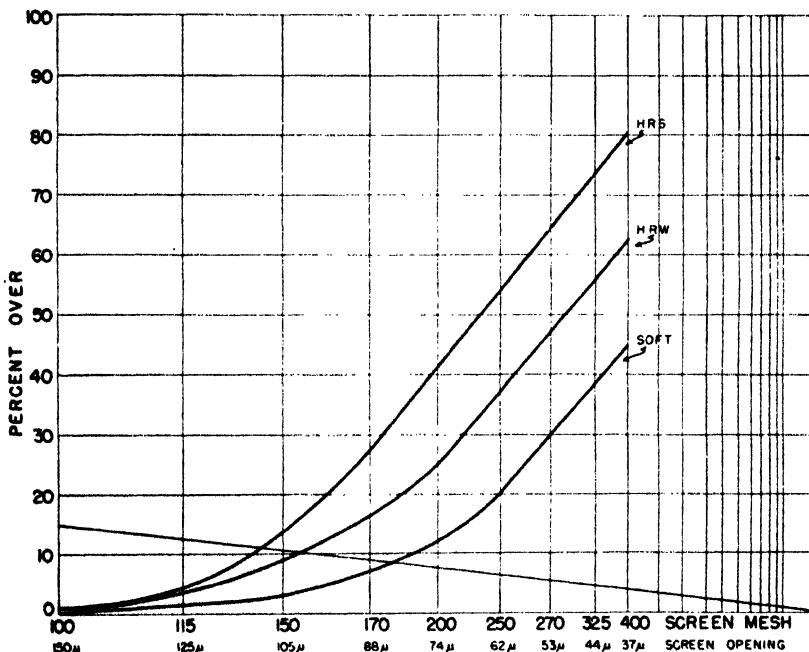


Fig. 2. Cumulative direct plot method for showing particle size distribution curves for flour.

density, but heterogeneous particle size, into fractions of a more uniform particle size. Stokes' Law relates the terminal velocity of fall:

$$V = \frac{10^{-8}g D d^2}{18n}$$

where V = terminal velocity of fall in cm./sec. in a stationary fluid,

g = constant of gravitation in cg. units = 980,

D = density of particle in g./ml.,

n = viscosity of fluid in cg. units = 1.82×10^{-4} for air,

d = diameter of particle in microns;

substituting in the above equation gives:

$$V = 0.00299 Dd^2 \text{ cm./sec.}$$

With a knowledge of the true density of the powder, D , an air velocity is adjusted corresponding to the settling velocity of the coarsest particle in the finest fraction to be obtained. The particles finer than the arbitrarily selected coarse limit of the fraction are floated up through an expansion chamber and are filtered from the air. Over-sized particles and agglomerates fall back into the U-tube, where the agglomerates are acted on by the jet of air and in the course of time are freed of their content of undersized particles. An increase in air velocity to that corresponding to the coarsest particle in the next desired fraction (Table II) will result in the removal of a fraction coarser than the coarse limit of the first fraction and finer than the coarse limit of the second fraction. Each succeeding increase in air velocity, depending on the size of the expansion chamber, removes a coarser

TABLE II
ROLLER AIR ANALYZER DATA FOR ADJUSTMENTS TO REMOVE THE
DESIGNATED FLOUR-FRACTION SIZES

Fraction size	Air velocity	Cylinder diameter	Nozzle orifice	Capillary orifice	Flowmeter setting
μ	$L/min.$	in.	in.	in.	in.
0-5	2.60	9	0.038	0.067	2.6
0-6	3.72	9	0.042	0.067	4.9
0-7	4.82	9	0.046	0.067	7.7
0-8	6.45	9	0.055	0.067	13.6
0-9	8.10	9	0.059	0.067	19.7
0-10	10.40	9	0.070	0.120	2.8
0-12	14.88	9	0.082	0.120	5.4
0-14	19.28	9	0.096	0.120	8.8
0-16	25.80	9	0.104	0.120	15.0
0-18	8.10	4 $\frac{1}{2}$	0.059	0.067	19.7
0-20	10.40	4 $\frac{1}{2}$	0.070	0.120	2.8
0-24	14.88	4 $\frac{1}{2}$	0.082	0.120	5.4
0-28	19.28	4 $\frac{1}{2}$	0.096	0.120	8.8
0-32	25.80	4 $\frac{1}{2}$	0.104	0.120	15.0
0-36	8.10	2 $\frac{1}{4}$	0.059	0.067	19.7
0-40	10.40	2 $\frac{1}{4}$	0.070	0.120	2.8
0-48	14.88	2 $\frac{1}{4}$	0.082	0.120	5.4
0-56	19.28	2 $\frac{1}{4}$	0.096	0.120	8.8
0-64	25.80	2 $\frac{1}{4}$	0.104	0.120	15.0
0-72	8.10	1 $\frac{1}{4}$	0.059	0.067	19.7
0-80	10.40	1 $\frac{1}{4}$	0.070	0.120	2.8
0-88	14.88	1 $\frac{1}{4}$	0.082	0.120	5.4
0-96	19.28	1 $\frac{1}{4}$	0.096	0.120	8.8
0-104	25.80	1 $\frac{1}{4}$	0.104	0.120	15.0

fraction from the sample until a complete sample fractionation is accomplished.

A 5-g. sample of flour was introduced into the U-shaped sample tube. An air velocity, adjusted by a flowmeter, was such that it would remove a predetermined size fraction. The separation end point was determined by calculating the rate of separation. The initial rate was taken as that amount of material removed in a 10-minute interval. When the rate of separation was one-tenth of the initial rate, the operation was considered to have reached the end point. Beyond this latter rate was the rate at which slightly oversized particles appeared due to the inception of a parabolic velocity gradient. Succeeding trials established end points of separation for various other size fractions. The weight of each fraction removed is calculated as per cent of the original sample weight. Table III gives the data for the hard red

TABLE III
PARTICLE SIZE DISTRIBUTION DATA BY THE
ROLLER AIR ANALYZER METHOD

Fraction size μ	Quantity of fraction separated	
	I	II
0-36	15.3	15.9
0-48	26.2	27.8
0-56	39.7	40.5
0-80	62.1	62.5
0-88	90.8	91.6
0-96	97.0	97.4

spring flour fractionated with the Roller Air Analyzer, with the particle size distribution curve shown in Fig. 3.

Pipette Sedimentation Method. The purpose of the present study was to attempt to select comparatively inexpensive simple sedimentation apparatus and to create conditions which would satisfy Stokes' Law so that the apparatus could be used for the entire particle size range of flour.

Sedimentation is the most frequently used process for the sizing analysis of particles normally below the size range of sieves. Many methods employing numerous types of equipment are described in the literature and have recently been reviewed by Stairmand (11), Heywood (4), Pieters and Hovers (9), and Davies (2).

It is necessary to begin sedimentation with a homogeneous mixture of flour particles and liquid medium. After a definite time lapse a variation in concentration of particles and medium will occur at a fixed depth below the surface. This concentration becomes a calculable function of particle size in Stokes' Law. The Andreasen pipette

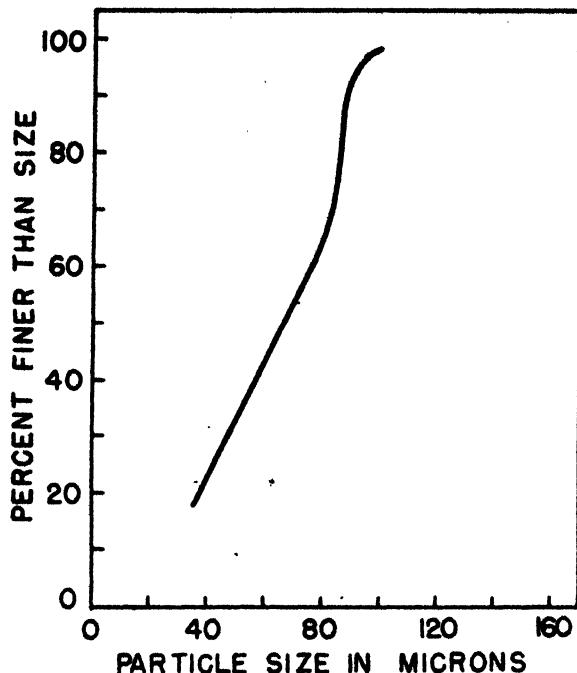


Fig. 3. Particle size distribution curve for wheat flour obtained by air separation.

apparatus was selected to determine this concentration by withdrawing small samples of the suspension. The Andreasen apparatus, shown in Fig. 4, consisted of a glass cylinder approximately 6 cm. in diameter with a capacity of 550 ml. when filled to the upper mark on the scale. It is provided with a ground glass stopper through which passes the stem of a pipette. The pipette extends 20 cm. beneath the surface of the suspension and 4 cm. from the cylinder bottom. The tip is at the level of the zero mark on the scale, while the upper surface of the suspension is at the 20 cm. mark. The pipette has a capacity of 10 ml. and is provided with a three-way stopcock and spout for draining into an evaporating dish. A uniform suction for withdrawing samples is provided by a water gravity-flow bottle.

Andreasen (1) found that Stokes' Law could be applied to angular or cubical particles of the same weight as spherical particles. By calculating the particle size as the edge length of a cube of the same volume as a sphere of radius r , his particle size conformed to the results of sieve analysis. This expression is shown as:

$$r = \left[2739 \sqrt{\frac{nh}{(D-d)tg}} \right] 1.612 \quad (A)$$

where r = edge length of particle in microns,

n = viscosity of suspending medium in poises,

h = height in cm. between liquid surface and pipette tip when sample is drawn,

g = gravitation constant (980.3 dynes),

t = time in minutes of settling,

D = density of flour,

d = density of suspending medium.

The suspending medium was a mixture of carbon tetrachloride and naphtha. Viscosity was determined by a Ubellhode viscosimeter. The specific gravity of the suspending medium was determined by the usual method with a pycnometer. Air buoyancy corrections were applied to all weights. Flour specific gravity was determined by the pycnometric method using naphtha as the required medium.

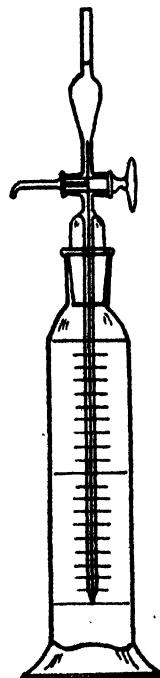


Fig. 4. Andreasen pipette sedimentation apparatus.

With the above values substituted into equation (A), the expression reduces to

$$t = \frac{k^2 h}{r^2}$$

where k is a constant for all the known values. The particle size (r)

TABLE IV
PARTICLE SIZE DISTRIBUTION DATA¹ FOR FLOUR BY
THE ANDREASSEN SEDIMENTATION METHOD

Calculated fraction size μ	Height of fall (h) cm.	Time of fall (t) min.	I		II	
			Residue recovered mg.	Finer than size %	Residue recovered mg.	Finer than size %
Sample	20.4	0	99.8	100.0	100.3	100.0
125	20.0	1.50	94.2	94.4	96.5	96.0
100	19.6	2.30	80.0	80.1	92.9	92.5
80	19.2	3.52	61.5	61.6	81.1	80.7
65	18.8	5.22	38.8	38.9	63.3	63.0
50	18.4	8.64	—	—	32.7	32.5
40	18.0	13.20	21.0	21.1	21.3	21.2
30	17.6	23.00	14.7	14.7	17.2	17.1
20	17.2	50.50	10.4	10.4	10.3	10.2

¹ Specific gravity of suspending medium = 1.309, flour = 1.438.

is chosen, the height of fall (h) is determined, and the time for the rate of fall for the particle size selected is calculated.

A 6-g. portion of the flour was introduced into the column. The

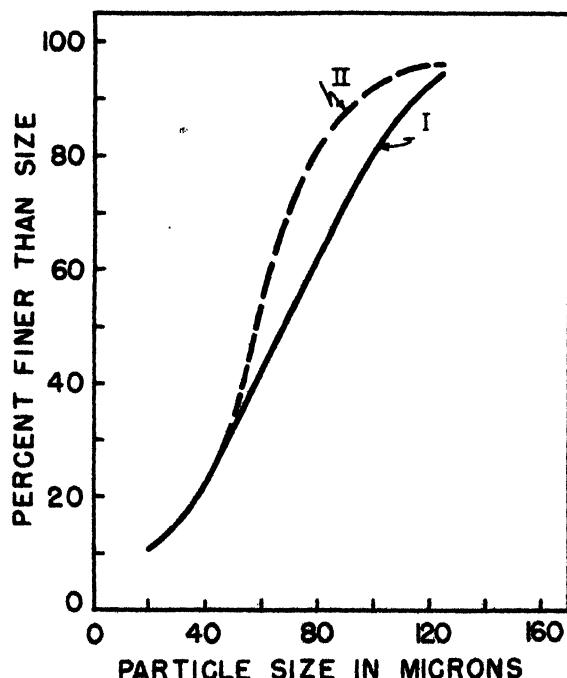


Fig. 5. Sedimentation curves obtained in duplicate trials with wheat flour illustrating the data in Table IV.

flour and medium were mixed thoroughly and an initial sample taken immediately. The apparatus was then placed in a constant temperature bath ($30^{\circ}\text{C.} \pm 0.1^{\circ}$) and subsequent samples withdrawn at pre-determined time intervals. Following discharge of the sample into a tared evaporating dish, the sample was dried and weighed. The data for a sedimentation trial on the hard red spring flour are given in Table IV, with the particle size distribution curves shown in Fig. 5.

Discussion

The means of data for sieving, air, and sedimentation analyses respectively are compared by the particle size distribution curves in Fig. 6.

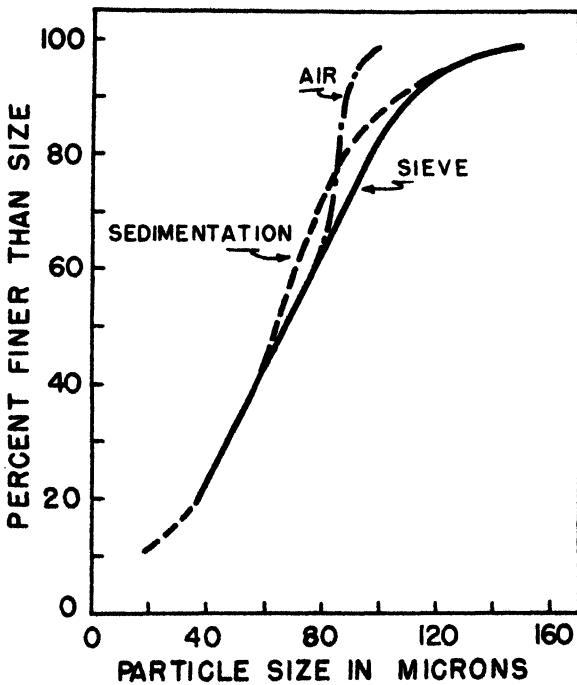


Fig. 6. Comparison of particle size distribution curves for wheat flour by sieving, air separation, and sedimentation.

The sieving test produces an accurately located curve for the entire size range of flour. Of the three methods, the apparatus required is the most applicable to the study of flour. The initial expense is moderate for the Ro-Tap and Tyler screens and the test requires less tedium for accurate data. For control work, it is not necessary to use all of the screens named. Four well-chosen screens are sufficient to produce data for an accurate particle size distribution curve. Many flours

were examined by the above method, with satisfactory results. The accuracy of the data is determined by a comparison with the results of other methods within their size range.

A stack sieve arrangement would shorten the sieving procedure somewhat if such a procedure would produce satisfactory data. Numerous attempts employing various sieve arrangements and cleaners were made. In no case were the data as reproducible as with the single sieve method. The variations in the curve characteristics by the stack arrangement were greater than the average differences of a vitreous and semivitreous wheat flour, thereby eliminating this arrangement as an accurate method of determining flour granulation.

The use of air for determining the particle size distribution in flour is an accurate method, but limited to the smaller size ranges. Fig. 6 shows that the air curve is in close agreement with the curve produced by sieving below the size of 80μ ; for larger particles Stokes' Law is not valid using the Roller Air Analyzer. In addition to the factor of viscous resistance embodied in Stokes' Law, there is an added resistance due to the changes in momentum of the air in contact with the falling particles. A correction to Stokes' Law is possible (Gonell, 3) by modifying the air flow or correcting the approximate air flow to account for the resistance due to the change in momentum of the air, making size separation with the particle size analyzer applicable up to 150μ .

The theory of particle size separation by air flow is based upon spherical particles. Flour particles, except starch granules, are angular and substantially spherical, deviating only slightly from Stokes' Law. Thus, theoretically and practically, air is a desirable medium for flour particle size separation, especially in the finer size ranges. The disadvantages of using the Roller apparatus are few besides the initial expense and limited size range. The method is rapid, making it possible to establish many accurate separation points.

The sedimentation data were selected at random among the many sets of similar data. Fig. 6 shows a close agreement of the sieving, air, and sedimentation data below the 50μ range, although poor agreement above this size. Many attempts were made by employing media of different specific gravities and employing refined sample withdrawal technique to overcome the pipette disadvantage of the Andreasen apparatus, where short time intervals for sample withdrawal in the large size ranges are encountered. Large errors are produced when the specific gravity of the medium approaches that of flour.

The disadvantages of the sedimentation method using the Andreasen apparatus are numerous. Exact calibration to obtain the physical

constants, as well as precise attention to details, is necessary to determine flour particle size. Also the apparatus must be used in a controlled temperature bath. Under the conditions used in this work the method gave poor data above the 50- μ size range.

Some advantages are seen in the sedimentation method employing other types of apparatus. Accuracy is obtained from apparatus employing a photo cell and galvanometer to determine the concentration gradient (6). The cumulative balance method was used successfully by Markley (7) and Hildebrand (5).

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HIGH LEVELS OF ALPHA-AMYLASE IN BAKING. I. EVALUATION OF THE EFFECT OF ALPHA-AMYLASE FROM VARIOUS SOURCES¹

JOHN A. JOHNSON and BYRON S. MILLER²

ABSTRACT

The use in the baking process of high levels of alpha-amylase from various sources has been studied together with means of measuring amylase activity.

Improved response to malt supplements by the addition of ball-milled starch to normal flour is possible without the detrimental effects on gas retention usually noted when flour itself is overground. The improvement was associated with saccharifying activity of the amylases during fermentation rather than the liquefying activity during the baking.

Fungal alpha-amylase was less effective than equivalent concentrations of malted wheat or barley alpha-amylase in reducing the maximum viscosity of gelatinized starch. This difference was associated with the relative temperatures of inactivation of the different alpha-amylases. Fungal alpha-amylase, however, was slightly more effective in increasing gas production and in yielding improvements in the bread denoted as malt response.

The recommendation for malt requirement of flour depends on the method used for its measurement.

The use of amylases in baking has been adequately reviewed recently by Kneen and Sandstedt (12) and by Geddes (5). Millers and bakers do not agree as to what constitutes satisfactory malt supplementation. Johnson, Shellenberger, and Swanson (7) showed that flours manufactured for a specific use exhibited wide variations in maltose value, gassing power, and maximum viscosity as measured by the amylograph. Since the different methods of evaluating alpha-amylase depend on various physical and chemical changes, it is not surprising that a desired supplementation level estimated by one method does not agree with the requirements when another method is used.

Recently the amylograph has been gaining steadily in popularity as a rapid means for controlling alpha-amylase supplementation. Selman and Sumner (15) contend that the amylograph results are more valuable than either maltose or gassing power determinations because results of starch liquefaction with the amylograph are more comparable with the actual baking process. Anker and Geddes (3), however,

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caution against a too liberal interpretation of maximum flour gelatinization viscosity as an index of alpha-amylase activity. Maximum viscosity was found by them to be affected by starch concentration and susceptibility, pH, and salts, in addition to alpha-amylase activity.

Alsberg and Griffing (1), Sherwood and Bailey (16), Karacsonyi and Bailey (9), Jones (8), and Bottomley (4) have shown that increased maltose and gassing power are associated with the extent of damage and rupture of starch granules. Selman and Sumner (15), on the other hand, show that overgrinding is not associated with a corresponding decrease in maximum viscosity of the gelatinized starch. It thus appears that the effect of malt supplementation and the influence of starch granule susceptibility thereon may depend upon the method of measurement.

Although one of the proposed standards of the regulations of the Federal Food, Drug, and Cosmetic Act lists as optional ingredients for bread, malt syrup, dried malt syrup, malted barley flour, and malted wheat flour, which are diastatically active, the use of alpha-amylase from sources other than wheat or barley merits consideration. It must be recognized, however, that the use of different sources of alpha-amylase presents special problems in measurement, due to the fact that the enzymes possess different properties as well as different thermal inactivation points (Kneen and Sandstedt, 12). Several investigators including Hollenbeck and Blish (6) and Olson, Burkhart, and Dickson (13) have shown that a single enzyme is responsible for both dextrinization and liquefaction of starch. This, however, does not exclude the possibility that the dextrinization and liquefaction methods for measuring alpha-amylase activity may give entirely different relative results when alpha-amylases from different sources are employed.

The present study was undertaken to investigate the utility in baking of high levels of alpha-amylase from malted wheat flour, malted barley, and mold bran, and to study different means of measuring their effects.

In addition, a study of the effect of excessive grinding on starch susceptibility and its relation to alpha-amylase response and control was included.

Materials and Methods

The principal flour used was a commercial straight grade, unbleached, unmalted sample, having a protein content of 12.5% and an ash content of 0.45%. This flour showed good malt response in baking. For the study of the effect of ball-milling on malt response, several flours of hard red winter wheat varieties were available. Each

of these flours was a blend of several smaller straight grade, experimentally milled samples. These blends had an average protein content of approximately 12.0% and an ash content of 0.45% (14% moisture basis).

The sources of alpha-amylase employed included a commercial malted wheat flour having an activity of 48 alpha-amylase units, a commercial malted barley, ground on a Hobart laboratory mill before using, and a fungal alpha-amylase material, which was a wheat bran cultured with *Aspergillus oryzae*.

A straight-dough procedure was employed in baking. Optimum mixing was determined by observing dough characteristics. A 3-hour fermentation at 30°C. and two intermediate punches with the National pup sheeter was used in all cases. Moulding was done with a Thompson laboratory moulder, the proof was for 55 minutes at 30°C., and the loaves were baked for 25 minutes at 425°F.

The baking formula was varied according to the requirements of the experiment as shown in the following:

Ingredient	Grams
Flour	100 (14% moisture basis)
Yeast	2.0
Salt	1.5
Sugar	3 to 6
Shortening	3.0
Milk	0 to 4
Potassium bromate	0.001 to 0.003
Alpha-amylase supplement	According to experiment
Water (0.2% calcium chloride solution)	As required

The malt supplements were prepared by extracting the enzymes from their sources with an 0.2% calcium chloride solution for one hour at 30°C. Kneen and Sandstedt (11) have demonstrated that such extracts are satisfactory means of malt supplementation and that this procedure lends itself to experimental manipulation of the enzyme.* Also, preliminary experiments with extracts of malted wheat flour gave results comparable to experiments with the original malted wheat flour. In studying the effect of varying enzyme concentration, a 1:10 extract of the enzyme source usually had sufficient alpha-amylase activity to permit making the desired dilutions with 0.2% calcium chloride solution.

The alpha-amylase activity of these extracts was determined by the modified Wohlgemuth dextrinization method, employing excess beta-amylase, as prescribed by Sandstedt, Kneen, and Blish (14).

The proper dilutions of the various sources of alpha-amylase were calculated to correspond to malted wheat flour as a reference. All

* It is a common practice of bakers to add yeast foods containing calcium.

extracts are thus expressed in terms of relative alpha-amylase activity, based on the activity of 0.25% malted wheat flour, considered as normal or 1X concentration.

The starch used in studies dealing with starch susceptibility to amylase was washed under tap water from dough of the same sample of flour used in the baking experiments. The starch was separated from the liquid by centrifugation, dried at room temperature on glass plates before a fan, and ball-milled for a period of 24 hours in a pebble mill, rotating approximately 80 r.p.m.

In experiments on the temperature of inactivation of alpha-amylase, the usual buffered salt solutions (sodium citrate-hydrochloric acid or dibasic sodium phosphate-citric acid) precipitated the calcium present in the enzyme extracts. A flour extract was therefore used as a buffer medium for carrying the amylase enzymes in these experiments. By this procedure it was possible to subject the amylases to an environment of pH similar to that in bread dough.

The buffer medium was made by suspending 130 g. of the commercial flour in 225 ml. of distilled water for one hour. The flour suspension was then centrifuged and the centrifugate heated to boiling to destroy all enzymes, to gelatinize the starch, and to coagulate the soluble proteins. Precipitated material was then removed by filtering through cotton. After cooling, 0.5 g. of diastase (Merck and Company) was added to digest the gelatinized starch. This solution, after standing for 2 hours, showed the absence of free starch by the iodine test. The solution was heated again to inactivate the added diastase. The solution had a pH of 5.6, was clear, but possessed a slight yellowish hue.

To determine the temperature of enzyme inactivation, equivalent amounts of the enzymes were added to 225 ml. of flour extract and the final volume made up to 450 ml. with water. The flour extract concentration was thus comparable to that of a flour-water suspension used for a normal amylograph curve. Sufficient calcium chloride was added to give a concentration of 0.2%. This was placed in the amylograph bowl. The temperature was increased at a uniform rate of 1.5°C. per minute. An aliquot of 5 ml. was withdrawn after each five degree rise in temperature and immediately transferred to test tubes standing in ice-water. Alpha-amylase activity was determined on these aliquots by the dextrinization method of Sandstedt, Kneen, and Blish (14).

The amylograph curves for flour were made according to the method described by Anker and Geddes (3) using 65 g. (14% moisture basis) of flour and 450 ml. of liquid. Gassing power tests were made according to *Cereal Laboratory Methods*, 4th ed. (2).

Results and Discussion

Effect of Added Ball-Milled Starch on Malt Response. Preliminary experiments with numerous flour samples showed little baking response to malt supplements whether a formula rich or lean in sugar was employed. Amylograph curves made with these flours gave maximum viscosities of 1,000 Brabender units or more, suggesting a low level of

TABLE I

EFFECT OF ADDED BALL-MILLED STARCH¹ ON RESPONSE TO ALPHA-AMYLASE FROM VARIOUS SOURCES IN BAKING

Enzyme source	Relative enzyme concentration ²	Loaf volume cc.	Grain ³ %	Texture %	Crust color	Break and shred
COMMERCIALLY MILLED FLOUR						
Control	0	675	80-o	75	Pale	Fair
Malted wheat	4X	698	80-o	77	Medium brown	Good
Malted wheat	12X	728	80-o	77	Dark brown	Very good
Malted barley	4X	698	83-o	79	Medium brown	Good
Malted barley	12X	745	85-o	82	Dark brown	Very good
Fungal	4X	735	83-o	75	Dark brown	Very good
Fungal	12X	755	90-o	93	Dark brown	Very good
EXPERIMENTALLY MILLED FLOUR						
Control	0	645	75-o	75	Pale	Fair
Malted wheat	4X	660	75-o	75	Light brown	Good
Malted wheat	12X	710	75-o	77	Dark brown	Very good
Malted barley	4X	663	80-o	77	Medium brown	Good
Malted barley	12X	705	85-o	82	Medium brown	Very good
Fungal	4X	685	80-o	75	Medium brown	Good
Fungal	12X	713	82-o	90	Dark brown	Very good

¹ 10 g. ball-milled starch substituted for 10 g. flour.

² 1X is equivalent to 0.25% malted wheat flour (48 alpha-amylase units) based on flour weight.

³ o = open.

inherent alpha-amylase activity or resistance to amylase attack. These samples, after ball-milling for 1, 2, 8, 12, and 24 hour periods, were baked with several increments of malted wheat flour. The data obtained corroborated those of Alsberg and Griffing (1) in that ball-milling even for a period as short as one hour was detrimental to baking quality. The absorption capacity was increased, the loaf volume was decreased, the grain and texture became open and harsh, and the crust color became a darker brown. These characteristics were accentuated

as time of ball-milling was increased up to 8 hours. No further effect appeared beyond this time.

To improve the amyloytic characteristics of the flour without affecting the colloidal and gas-retaining properties as a dough, starch extracted from the flour samples was ball-milled for a 24-hour period and 10 g. of this starch were substituted for 10 g. of the flour.⁴ Representative data from the baking tests with various concentrations of malted wheat flour, malted barley, and fungal alpha-amylase are presented in Table I. Both the commercially and experimentally milled flour responded to alpha-amylase supplements with the addition of ball-milled starch. Loaf volume, grain texture, crust color, and external appearance improved with increments of alpha-amylase supplements as high as an equivalence of 12 times the normal malt dosage. There was, furthermore, no slackening or stickiness in the doughs even at these high levels of alpha-amylase supplementation. The beneficial effects of wheat and barley alpha-amylase were similar but somewhat less marked than that of fungal alpha-amylase.

The influence of ball-milled starch on the maximum viscosity of flour paste in the presence of alpha-amylase is shown in Table II.

TABLE II
INFLUENCE OF ADDED BALL-MILLED STARCH ON MAXIMUM VISCOSITY
OF FLOUR PASTES IN PRESENCE OF ALPHA-AMYLASE

Flour	Ball-milled starch	Maximum viscosity	
		No malt supplement	0.25% malted wheat flour
6	6	B.U. ¹	B.U. ¹
65	0	875	290
60	5	880	260
55	10	870	255
50	15	880	250

¹ B.U. = Brabender units.

The addition of ball-milled starch had little effect on the maximum viscosity. It is significant, however, that the ball-milled starch did not tend to increase the maximum viscosity as might be expected if raw starch was added to the flour. Ball-milled starch in the flour slightly decreased the maximum viscosity in the presence of 0.25% malted wheat flour; however, the amount of this decrease was not so great as was expected from the baking results. These data corroborated those of Selman and Sumner (15).

It would appear from the data in Tables I and II that the presence

⁴ It is recognized that some bakers have followed the practice of adding solubilized starch to increase effectiveness of malt products.

of injured starch granules would affect the recommendation of "malt" requirement based on methods used to measure saccharifying activity, but would have little effect on the recommendations if based on amylograph data. The addition of ball-milled starch to flour is beneficial to amylase activity, and no detrimental effects appeared in gas retention.

Influence of Concentration of Alpha-Amylase from Various Sources on Maximum Viscosity. The influence of equivalent alpha-amylase concentrations from different sources on the maximum flour paste viscosity is shown in Table III. Malted barley alpha-amylase appears

TABLE III

INFLUENCE OF ALPHA-AMYLASE CONCENTRATION AND SOURCES UPON
MAXIMUM VISCOSITY OF GELATINIZED FLOUR PASTE

Relative enzyme concentration ¹	Maximum viscosity
Control	B.U. ² 1000
<i>Malted wheat flour alpha-amylase</i>	
1X	450
12X	120
24X	50
<i>Malted barley alpha-amylase</i>	
1X	275
12X	90
24X	50
<i>Fungal alpha-amylase</i>	
1X	880
12X	720
24X	550

¹ 1X is equivalent to 0.25% malted wheat flour.

² B.U. = Brabender units.

to be the most effective in reducing the maximum viscosity, being followed closely by malted wheat flour. The fungal alpha-amylase was much less effective.

Comparative thermo-stabilities of the alpha-amylase from the three sources, as determined by methods already described, are shown in Table IV, in terms of the per cent of alpha-amylase activity remaining when the indicated temperature has been reached in the amylograph. Barley alpha-amylase was most resistant to thermal inactivation, being closely followed by malted-wheat alpha-amylase. Fungal alpha-amylase was the most susceptible to inactivation by heat. These data are in agreement with the observations of Hollenbeck and Blish (6). Inactivation was a gradual process, as shown by the fact that activity decreased over a relatively wide range of temperature.

Since the most active period of liquefaction by alpha-amylase is in the temperature range of starch gelatinization, the maximum viscosity

TABLE IV
EFFECT OF TEMPERATURE UPON THE INACTIVATION
OF ALPHA-AMYLASE FROM DIFFERENT SOURCES

Temperature	Malted wheat flour alpha-amylase activity remaining	Malted barley flour alpha-amylase activity remaining	Fungal alpha-amylase activity remaining
°C.	%	%	%
60	100	100	100
65	97.9	100	100
70	91.0	100	63.0
75	29.1	71.9	6.3
80	14.5	29.1	0.0

is controlled to a large extent by the temperature at which the particular enzyme is inactivated. Thus the higher the temperature of inactivation of the alpha-amylase, assuming equivalent concentration, the greater would be its activity as measured by the amylograph. The differences in the maximum viscosity for the three alpha-amylase sources employed in this study may therefore be explained by the differences in thermal inactivation.

Effect of Malted Wheat Flour and Fungal Alpha-Amylase upon Gassing Power. Data showing the gassing power obtained from various amounts of malted wheat flour and fungal alpha-amylase are shown in Table V. Above 12X concentration of malted wheat flour alpha-amylase there was no increase in gassing power, but with fungal alpha-amylase there was a slight increase up to 24X concentration. The

TABLE V
EFFECT OF MALTERD WHEAT FLOUR AND FUNGAL ALPHA-
AMYLASE UPON GASSING POWER

Relative enzyme concentration ¹	Hours		
	1	6	24
Control	mm. 86	mm. 343	mm. 539
MALTERD WHEAT FLOUR ALPHA-AMYLASE			
1X	78	489	755
12X	107	710	1212
24X	110	696	1207
FUNGAL ALPHA-AMYLASE			
1X	102	596	980
12X	103	687	1213
24X	109	724	1346

¹ 1X is equivalent to 0.25% malted wheat flour.

effect of fungal alpha-amylase on gassing power at 12X concentration was equal to, and at 24X concentration even greater than, that of malted wheat flour alpha-amylase. These effects are opposite to those measured with the amylograph and suggest that recommendations of the malt requirement of a flour would vary, depending on the method employed to measure malt response.

Effect of Malted Wheat Flour and Fungal Alpha-Amylase Concentration on Baking Results. The amylograph technics may simulate the effect which alpha-amylase might have upon the starch during the baking process, while gassing power is related to the amylase effects during the fermentation process. The baking test, however, is the final criterion of proper malt supplementation. The data in Table VI

TABLE VI
EFFECT OF MALTED WHEAT FLOUR AND FUNGAL ALPHA-AMYLASE CONCENTRATION ON BAKING RESULTS

Relative enzyme concentration ¹	Loaf volume	Grain		Texture
		cc.	%	
0	715		80-0	80
MALTED WHEAT FLOUR ALPHA-AMYLASE				
1X	760	83-0		85
12X	830	85-0		88
24X	850	88-0		92
FUNGAL ALPHA-AMYLASE				
1X	760	82-0		85
12X	850	88-0		95
24X	880	85-0		92

¹ 1X is equivalent to 0.25% malted wheat flour.

show baking effects of malt supplements at high levels. The baking was done with a formula containing 6% sugar so that adequate yeast nutrition was provided without dependence on sugar formation by amylase action. Both malted wheat flour and fungal alpha-amylase increased the loaf volume and improved other characteristics of the bread.

It would be anticipated from the work of Kozmin (10) that the high concentrations of alpha-amylase employed in this study would cause excessively wet and sticky doughs and soggy bread crumb. From the amylograph results it might also be expected that excessive starch liquefaction would occur with 12X and 24X concentrations of malted wheat flour as compared with equivalent concentrations of

fungal alpha-amylase. There was, however, no evidence of stickiness of dough or a soggy bread crumb with either malted wheat flour or fungal alpha-amylase.⁵

From the baking data (Table VI) employing the straight dough procedure it would appear that the upper safe limit of malt supplementation could exceed 24 times the normal concentration of 0.25% malted wheat flour or equivalent amounts of alpha-amylase from fungal or barley sources. The safe maximum limit will, of necessity, vary with the thermo-stability of the alpha-amylase. It would appear that if the alpha-amylase source has an exceedingly high inactivation temperature, detrimental effects would be obtained with large dosages. The concentration and temperature of inactivation of the alpha-amylases employed in these studies were not high enough to observe any detrimental effects on baking, yet the concentrations were 24 times greater than normally used.

Acknowledgments

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⁵ It is recognized that failure to obtain these adverse effects of high concentration of alpha-amylase may be due, in part, to the particular baking procedure employed. This will be discussed further in the second paper of this series.

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HIGH LEVELS OF ALPHA-AMYLASE IN BAKING. II. PROTEOLYSIS IN STRAIGHT AND SPONGE DOUGHS¹

BYRON S. MILLER and JOHN A. JOHNSON²

ABSTRACT

The use of high levels of alpha-amylase in both the straight and sponge dough baking procedures was investigated. Malt supplements containing excessive amounts of proteolytic enzymes, such as an extract of a fungal bran preparation, caused soft and sticky doughs, inferior grain and texture, and extremely low loaf volume when the sponge procedure was used. No harmful effects were noted when using the straight dough procedure.

Removal of proteolytic enzymes from amyloytic preparations by adsorption on kaolin indicated that the detrimental action produced by high levels of certain malt supplements in baking was due to their inherent proteolytic capacity.

Sodium chloride acted as a proteolytic inhibitor, but potassium bromate had no inhibitory action. The addition of sodium chloride to the sponge in the sponge baking procedure permitted the use of high levels of malt supplements containing proteolytic enzymes with no harmful effects. The use of high concentrations of alpha-amylase in the sponge procedure as well as in the straight dough procedure was practicable and would permit the use of smaller sugar concentrations in the dough.

Detimental effects of certain malts and malt supplements have frequently been encountered and bakers are reluctant to add more than a very small percentage to their doughs. The literature, as reviewed by Hildebrand and Burkert (6), presents two conflicting theories on the cause of this detrimental action. The older theory proposed by Ford and Guthrie (2) postulates that the detrimental action is due to high proteolytic activity. The later theory originally proposed by

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Kozmin (8) and amplified by other writers including Hildebrand and Burkert (6), Geddes, Hildebrand, and Anderson (3), and Stamberg and Bailey (13) suggests that the increase in dough mobility and stickiness with the addition of excessive dosages of malt flour is due to the alpha-amylase activity of such malts rather than to their proteolytic activity. Kozmin (8) contended that when the alpha-amylase activity was excessively high the defects in the crumb were due to the starch degradation, producing dextrins which possessed decreased water-retaining capacity.

Johnson and Miller (7) showed that high levels of alpha-amylase may be used in straight doughs without the serious detrimental effects observed by Kozmin (8) and others. Read and Haas (10) noted that the action of certain enzyme preparations was more marked with the sponge method than with the straight dough baking procedures.

The detrimental effects ascribed to the presence of proteases in malts by earlier workers naturally led to attempts to remove them. Tissue and Bailey (14) concluded that proteolytic enzymes are almost completely removed from malted wheat preparations by precipitation with safranine. Read and Haas (11), however, were unable to effect a strictly quantitative removal of the proteases by this procedure.

Young and Hartman (15) found that several adsorbents including bauxite, permutite, kaolin, alumina, and pumice would remove trypsin from the amylolytic and lipolytic enzyme systems of pancreatic juice. Hemmi and Inami (4, 5) found that kaolin could be used for partially separating protease from the amylase of takadiastase and pancreatin preparations.

The objective of the present investigation was to study the use of high levels of alpha-amylase in baking with particular attention being directed to differences in proteolysis in straight and sponge dough baking procedures. Means of inhibiting proteolysis in sponge doughs and procedures for differentially separating proteolytic enzymes from amylolytic enzymes were also investigated.

Materials and Methods

The flour used in this study was the commercial straight grade, unbleached, unmalted sample having a protein content of 12.5% and an ash content of 0.45% (14.0% moisture basis) (Johnson and Miller, 7). This flour showed a good malt response in baking.

Two sources of alpha-amylase, a malted wheat flour and a fungal bran preparation, were employed.

The straight dough baking procedure employed by Johnson and Miller (7) was followed in the present study. All loaves were baked in

duplicate. In the sponge baking method the following formula was used:

	Sponge g.	Dough g.
Flour	70 (14% moisture basis)	30 (14% moisture basis)
Yeast	2	0
Sugar	0	4
Salt	1.5	1.5
Milk	—	4
Shortening	—	3
Potassium bromate	—	0.003
Alpha-amylase (added in extract form)	According to experiment	
Water (0.2% calcium chloride solution)	60% of total	40% of total

The sponge was mixed for 1½ minutes, given a 4-hour sponge time, 30-minute floor time, 20-minute rest before panning, 55-minute proof time, and baked for 25 minutes at 425°F. The fermentation temperature was 30°C. The dough mixing time was varied to obtain optimum dough development and handling properties. All loaves were baked in duplicate.

Enzyme extracts were used exclusively and were prepared by a one-hour extraction (30°C.) of the original alpha-amylase source with 0.2% calcium chloride solution. Dilutions were also made with 0.2% calcium chloride solution. Alpha-amylase determinations and adjustments were made according to the procedure of Sandstedt, Kneen, and Blish (12). Determinations of proteolytic activity were made using an Ayre-Anderson procedure as modified by Miller (9). Gassing power tests were made by the pressuremeter method described in *Cereal Laboratory Methods*, 4th ed. (1).

In both baking procedures the enzyme concentrations used ranged from 0 to 24 times the normal concentration of alpha-amylase provided by 0.250 g. of malted wheat flour (48 alpha-amylase units) per 100 g. of flour. In the text and tables the enzyme concentrations carry the notation 1X, 12X, or 24X to indicate that the amount of enzyme used is 1, 12, or 24 times the normal amount.

Results

Baking Effects of High Malt Levels in Straight and Sponge Doughs. Table I presents the data obtained from both straight dough and sponge procedures. All samples were baked on the same day. The one-hour difference in the straight and sponge dough procedures in no way accounts for differences in dough and loaf characteristics.

By the straight dough procedure the loaf volume and crumb characteristics improved with increasing alpha-amylase concentrations up

TABLE I

COMPARISON OF THE STRAIGHT AND SPONGE DOUGH BAKING PROCEDURES EMPLOYING VARIOUS LEVELS OF MALTED WHEAT FLOUR AND FUNGAL ALPHA-AMYLASE

Source of alpha-amylase	Relative ¹ enzyme concentration	Sugar %	Loaf characteristics			
			Loaf volume ml.	Grain ² %	Texture %	Break & shred ³
STRAIGHT DOUGH PROCEDURE						
Wheat malt	0	6	750	85-o	90	VG
Wheat malt	1X	6	755	85-o	90	VG
Wheat malt	12X	6	825	88-o	93	VG
Wheat malt	24X	6	825	89-o	93	VG
Wheat malt	0	3	670	83-c	80	F
Wheat malt	1X	3	685	85-c	82	G
Wheat malt	12X	3	755	90-c	86	G
Wheat malt	24X	3	775	90-c	90	VG
Fungal	1X	6	720	85-c	86	G
Fungal	12X	6	850	90-o	90	VG
Fungal	24X	6	870	93-c	95	G
Fungal	1X	3	675	83-c	80	F
Fungal	12X	3	785	86-o	85	VG
Fungal	24X	3	810	89-o	90	VG
SPONGE DOUGH PROCEDURE						
Wheat malt	0	4	685	87-c	85	F
Wheat malt	1X	4	710	91-c	90	G
Wheat malt	12X	4	760	85-c	85	G
Wheat malt	24X	4	810	83-o	84	F
Fungal	1X	4	720	90-c	87	G
Fungal	12X	4	675	75-o	70	P
Fungal	24X	4	580	50-o	50	P

¹ 1X = normal concentration of alpha-amylase equivalent to that provided by 0.250 g. of malted wheat flour per 100 g. of flour.

² c = close, o = open.

³ VG = very good, G = good, F = fair, P = poor.

to 24X. The volume obtained when using 6% sugar was appreciably larger than that obtained when only 3% sugar was used with comparable enzyme concentrations. The addition of either malted wheat flour at levels up to 6 g. per 100 g. flour (24X concentration of alpha-amylase) or a 0.2% calcium chloride extract of the same malt supplement containing an equivalent concentration of alpha-amylase produced doughs with similar handling properties and identical loaf volumes. The grain and texture and crumb color, however, were inferior when the malted wheat flour itself was used at 24X concentration.

By the sponge baking procedure the use of malted wheat flour gave increases in loaf volume with increasing alpha-amylase concentration, but the crumb properties were adversely affected. When fungal amy-

lase was used, the loaf volume and the crumb properties became inferior with increasing concentrations of fungal alpha-amylase. Likewise, the dough became sticky and exceedingly difficult to handle. The probable cause of the variation in results obtained with malted wheat flour and mold bran was realized when it was found that the fungal extract showed eight times the proteolytic activity of an equivalent amount of malted wheat flour extract based on alpha-amylase content.

Inhibition of Proteolysis in Sponge Doughs by Sodium Chloride. The striking difference noted between the loaves baked by the sponge and straight dough methods (Table I) was confirmed by further tests.

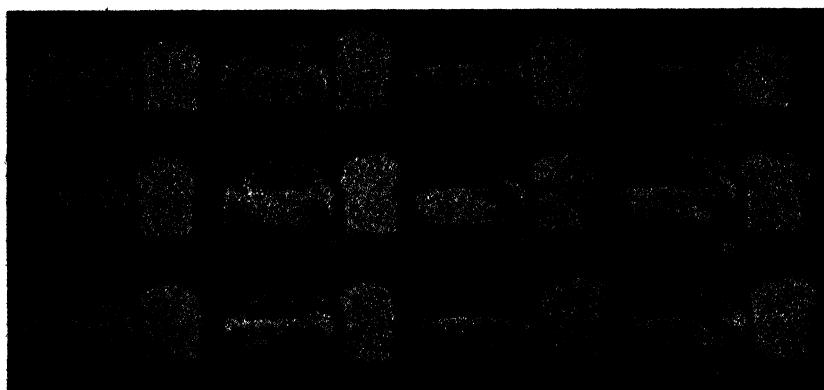


Fig. 1. Effect of sodium chloride in sponge doughs containing high levels of fungal alpha-amylase.

Alpha-amylase concentration	0	1X	12X	24X
No salt	1	2	3	4
1.05% salt	5	6	7	8
1.5% salt	9	10	11	12

Since only yeast and water normally are added to the sponge it was assumed that one or more of the baking ingredients normally added at the dough stage (straight dough procedure) prevented the appearance of the detrimental properties observed in the sponge.

Experiments were performed in which sodium chloride and potassium bromate were added both to the sponge and to the dough in the sponge baking procedure. The beneficial effects of adding sodium chloride to the sponge are shown by the baking data in Table II and by the photographs in Fig. 1. That potassium bromate did not restrain the detrimental action of high malt concentrations is shown in Table III and pictorially in Fig. 2.

Chemical data (Table IV) supporting the evidence provided by actual baking tests indicate that sodium chloride inhibits proteolytic enzymes while potassium bromate has no inhibitory effect. Table III and Fig. 2 also indicate that potassium bromate does not inhibit proteolytic activity during baking and that the optimum level of potassium bromate (3 mg. in this case) is independent of the concentration of alpha-amylase used or of the amount of proteolysis.

Removal of Proteolytic Enzymes. The data in Tables II and IV and Fig. 1 suggest that the detrimental effects observed in the sponge

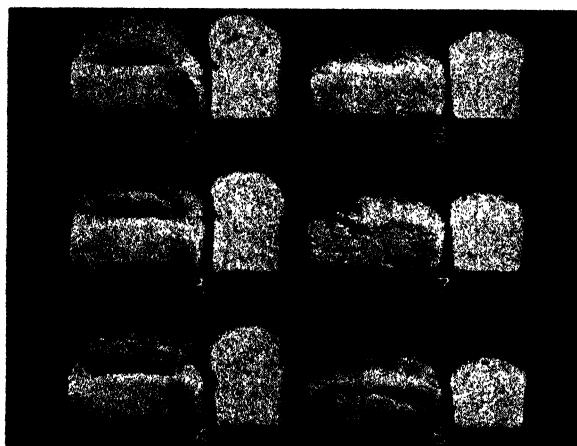


Fig. 2. Effect of potassium bromate in sponge doughs containing high levels of fungal alpha-amylase.

Alpha-amylase concentration	1X	24X
3 mg. % KBrO ₃	2	6
10 mg. % KBrO ₃	3	7
20 mg. % KBrO ₃	4	8

method are due to excessive proteolysis. This view is further substantiated by the data in Table III and Fig. 2, which indicate that the detrimental effects of high malt concentrations are not associated with the presence of an excess of reducing matter which might have an effect similar to that produced by proteolytic enzymes on the physical dough characteristics. This is shown by the fact that high concentrations of potassium bromate failed to improve bread characteristics in the presence of excess fungal extracts. As determined by titration with 0.1 *N* standard iodine and 0.1 *N* sodium thiosulfate solutions, the malted wheat flour extract actually contained about four times the quantity of reducing matter as an equivalent concentration of fungal alpha-amylase.

TABLE II
EFFECT OF SODIUM CHLORIDE IN SPONGE DOUGHS CONTAINING
HIGH LEVELS OF FUNGAL ALPHA-AMYLASE

Loaf no.	Relative enzyme concentration ¹	NaCl added to sponge	NaCl added to dough	Dough mixing time	Loaf characteristics				Dough handling at pan
					Volume	Grain ²	Texture	Break & shred ³	
1	0	0	1.5	2 min.	705	80-o	85	F	Good
2	1X	0	1.5	2	780	88-o	90	G	Good
3	12X	0	1.5	1	745	75-o	85	P	Slightly soft
4	24X	0	1.5	0.25	610	60-o	70	VP	Very soft
5	0	1.05	0.45	2	765	82-o	86	G	Good
6	1X	1.05	0.45	2	795	88-o	90	G	Good
7	12X	1.05	0.45	2	860	88-o	90	F	Good
8	24X	1.05	0.45	2	865	84-o	88	F	Slightly soft
9	0	1.5	0.0	2	770	82-o	85	G	Good
10	1X	1.5	0.0	2	785	86-o	88	G	Good
11	12X	1.5	0.0	2	850	86-o	90	F	Good
12	24X	1.5	0.0	1.75	860	83-o	88	F	Good

¹ 1X = normal concentration of alpha-amylase equivalent to that provided by 0.250 g. of malted wheat flour per 100 g. of flour.

² o = open.

³ G = good, F = fair, P = poor, VP = very poor.

TABLE III
EFFECT OF POTASSIUM BROMATE ON SPONGE DOUGHS CONTAINING
HIGH LEVELS OF FUNGAL ALPHA-AMYLASE

Loaf no.	Relative enzyme concentration ¹	KBrO ₃ in sponge	Total KBrO ₃	Dough mixing time	Loaf characteristics				Dough handling at pan
					Volume	Grain ²	Texture	Break & shred ³	
1	1X	0	3	2	780	88-o	90	G	Normal
2	1X	3	3	2	860	85-o	88	VG	Normal
3	1X	10	10	1.75	815	82-o	85	VG	Normal
4	1X	20	20	1.75	795	78-o	80	G	Normal
5	24X	0	3	0.25	610	60-o	70	VP	Very soft
6	24X	3	3	0.25	650	70-o	70	VP	Very soft
7	24X	10	10	0.25	580 ²	60-o	60	VP	Very soft
8	24X	20	20	0.25	505 ²	50-o	40	VP	Very soft

¹ 1X = normal concentration of alpha-amylase equivalent to that provided by 0.250 g. of malted wheat flour per 100 g. of flour.

² o = open.

³ G = good, VG = very good, VP = very poor.

Initial attempts to remove the proteolytic enzymes from amylase preparations were made using safranine dye and the procedure of Tissue and Bailey (14). This procedure was unsatisfactory, however, since the proteolytic activity could only be reduced 70% and a rather

TABLE IV

EFFECT OF SODIUM CHLORIDE AND POTASSIUM BROMATE ON PROTEOLYTIC ACTIVITY OF FUNGAL EXTRACT

NaCl	KBrO ₃	Fungal extract	Nitrogen/10 mg. extract	Inhibition
mg.	mg.	mg.	mg.	%
85 ¹	—	25	9.4	0
—	5.7 ¹	25	5.3	54
		25	9.4	0

¹ The quantities of salts used are in proportion to the amount used in baking.

large proportion of the alpha-amylase was simultaneously removed from the preparation.

To demonstrate more clearly the phenomena observed in the sponge procedure a method was developed to separate the proteolytic enzymes from alpha-amylase. A 25-ml. portion of fungal extract (1/10 dilution of the original extract) was added to kaolin (pH 7.3) and stirred periodically during a 30-minute standing time. The suspension was filtered under reduced pressure and the filtrate was checked for proteolytic and

TABLE V

REMOVAL OF PROTEOLYTIC ENZYMES FROM A FUNGAL AMYLOLYTIC PREPARATION USING KAOLIN AS AN ADSORBENT

Weight of kaolin ¹	Reaction	Enzyme activity of filtrate ²	
		Amyloytic activity retained	Proteolytic activity retained
0	pH 4.7	% 100	% 100
0.25	4.7	100	95
0.50	4.7	100	88
1.0	4.7	93	70
2.0	4.7	60	25
2.0	7.3	60	6

¹ Added to a 25-ml. portion of fungal extract (30 minutes contact time).

² The equivalent of 1.5 mg. mold bran used for the amyloytic determination and 10 mg. for the proteolytic determination.

amylolytic activity. The kaolin proved to be effective in removing proteolytic enzymes, but this was accompanied by adsorption of considerable alpha-amylase. The data in Table V show the effectiveness of kaolin for removing proteolytic enzymes from a fungal amylase preparation.

Baking data showing the effect of removal of proteolytic enzymes from a fungal alpha-amylase preparation by adsorption on kaolin (pH 7.3) are presented in Table VI. A photograph of the loaves is shown in Fig. 3.

TABLE VI

EFFECTS OF REMOVING PROTEOLYTIC ENZYMES FROM THE FUNGAL ALPHA-AMYLASE PREPARATIONS USED IN BOTH THE SPONGE AND STRAIGHT DOUGH BAKING PROCEDURES

Loaf no.	Relative enzyme concentration ¹	Proteolytic enzymes	Mixing time min.	Loaf characteristics				
				Volume ml.	Grain ² %	Texture %	Crumb color %	Break & shred ³
STRAIGHT DOUGH PROCEDURE								
1	0		3.0	800	80-o	83	86	G
2	1X	Present	3.0	785	83-o	85	90	G
3	12X	Present	3.0	825	88-c	87	90	VG
4	24X	Present	2.5	860	88-c	92	90	VG
5	1X	Removed	3.0	760	83-o	83	88	G
6	12X	Removed	3.0	825	83-o	83	88	VG
7	24X	Removed	3.0	850	86-o	85	90	VG
SPONGE DOUGH PROCEDURE								
8	0		2.0	745	80-o	83	86	G
9	1X	Present	2.0	790	80-o	83	86	G
10	12X	Present	1.0	750	75-o	78	82	P
11	24X	Present	0.5	660	60-o	70	70	VP
12	1X	Removed	2.0	785	88-o	85	88	G
13	12X	Removed	2.0	820	88-c	88	90	VG
14	24X	Removed	1.5	830	80-c	90	90	VG

¹ 1X = normal concentration of alpha-amylase equivalent to that provided by 0.250 g. of malted wheat flour per 100 g. of flour.

² o = open, c = close.

³ G = good, VG = very good, P = poor, VP = very poor.

TABLE VII

EFFECT OF PROTEOLYTIC ENZYMES ON THE GAS-PRODUCING CAPACITY OF A FLOUR CONTAINING HIGH LEVELS OF FUNGAL ALPHA-AMYLASE

Relative enzyme concentration ¹	Proteolytic enzymes	Hours		
		1	6	24
		Manometric pressure		
0	Present	mm.	mm.	mm.
		86	343	538
12X	Present	87	561	846
12X	Removed	90	568	857
24X	Present	90	614	989
24X	Removed	86	579	932

¹ 1X = normal concentration of alpha-amylase equivalent to that provided by 0.250 g. of malted wheat flour per 100 g. of flour.

The removal of proteolytic enzymes caused improvement in the loaf characteristics, mixing properties, and in loaf volume by both

the straight and sponge dough procedures. This improvement in the loaves baked by the sponge process with an enzyme preparation freed of proteolytic enzymes by adsorption is striking in comparison with the deterioration of the loaves baked by the same process with the original alpha-amylase preparation.

Effect of Proteolytic Enzymes on Gassing Power. The presence or absence of natural proteolytic enzymes in fungal amylase extracts

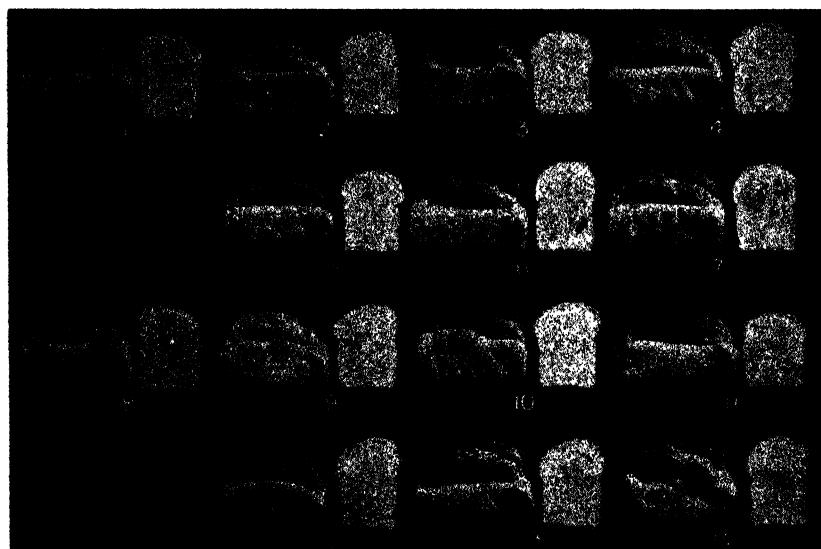


Fig. 3. Effects of removing proteolytic enzymes from the fungal alpha-amylase preparations used in both the sponge and straight dough baking procedures.

Alpha-amylase concentration	0	1X	12X	24X
Straight dough				
Proteolytic enzymes present	1	2	3	4
Proteolytic enzymes removed	—	5	6	7
Sponge dough				
Proteolytic enzymes present	8	9	10	11
Proteolytic enzymes removed	—	12	13	14

is shown by the data in Table VII to have no marked effect on the gas production capacity of the flour. Gas retention, on the other hand, was notably influenced as shown by reduction in loaf volume and the deterioration in bread characteristics recorded in Table VI. The effect of proteolytic enzymes on gluten also was evidenced by the stickiness of the doughs and by the very short re-mix times required for the sponge.

Discussion

The data clearly indicate the marked difference in the effect of proteolytic enzymes on the straight and sponge procedures. When high concentrations of alpha-amylase extracts containing large amounts of proteolytic enzymes were used in the sponge procedure, the re-mix time was reduced, the dough became extremely soft and sticky, the loaf volume was greatly decreased, and loaf and crumb characteristics became inferior. In the straight dough procedure, large concentrations of a similar preparation caused no harmful effects and even improved the quality of the bread.

Most authors suggest that the increase in mobility and stickiness resulting from large dosages of amylase preparations is due to excessive alpha-amylase activity rather than to proteolytic activity. The present work, however, indicates that malt extracts having high proteolytic activity are undesirable as baking adjuncts due to their effect on "gas retention capacity," as well as to their detrimental effects on physical dough properties. This appears to substantiate the idea of Ford and Guthrie (2).

The use of equivalent quantities of fungal and wheat malt alpha-amylase in the sponge procedure (Table I) reveals a sharp decline in loaf volume and crumb characteristics with increasing levels of fungal alpha-amylase. In contrast to this, the bread quality steadily improved with equivalent quantities of wheat malt alpha-amylase up to 12X. This difference in the action of the two supplements may be explained by the fact that the fungal preparation had eight times as much proteolytic activity as an equivalent amount of malted wheat flour alpha-amylase. When a large portion of the proteolytic activity was removed from the extract of fungal amylase by adsorption on kaolin, the baking results (Table VI) were similar to those obtained when malted wheat flour was used. An increase in loaf volume and an improvement in crumb properties were then obtained with increasing concentrations of the fungal alpha-amylase extract.

The data in Table II indicate that the addition of sodium chloride to the sponge tends to equalize the two baking procedures with respect to proteolytic activity. The deleterious effect of large additions of fungal amylase on loaf volume, handling, and crumb characteristics was counteracted by the addition of salt to the sponge. It made little difference whether the entire amount of sodium chloride was included in the sponge or whether a proportionate amount was added to the sponge and to the dough. This technique of adding a part of the salt to the sponge when the flour shows stickiness at the dough stage is a fairly common bakeshop practice.

The inhibition of fungal proteolytic enzymes by sodium chloride as determined by the modified Ayre-Anderson procedure (9) was found to be approximately 55% when using a concentration of salt equivalent to that present in the water added to the sponge dough. The data in Table III show that potassium bromate does not influence proteolytic action and that the optimum bromate level is 3 mg. whether 1X or 24X concentration of fungal amylase is used. Chemical determinations (Table IV) using the modified Ayre-Anderson procedure (9) confirm the failure of potassium bromate to influence proteolytic action. These data would appear to support the opinion that potassium bromate acts directly upon the gluten proteins and is not associated with the inhibition of proteolytic enzymes.

The possibility of using high levels of alpha-amylase to supplant, under emergency conditions, part of the sugar normally added in the straight dough procedure is suggested by the data in Table I. When either malted wheat flour or fungal alpha-amylase at 24X concentration was used in conjunction with only 3% sugar, the results were superior to those obtained from 1X concentration of enzyme and 6% sugar.

Further, the data in Table VI indicate that it is possible to use high levels of alpha-amylase in the normal sponge procedure when no salt is added to the sponge, provided the proteolytic enzymes are removed from the amylolytic preparation. Bread of high quality was produced by both straight dough and sponge procedures when using concentrations up to 24X of alpha-amylase, from which the proteolytic enzymes had been removed.

Acknowledgments

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ACTION OF MOLD ENZYMES IN STARCH SACCHARIFICATION¹

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ABSTRACT

Alcohol yields from fermentation of corn mashes saccharified with mold culture filtrates correlated more closely with the potency of a glucogenic enzyme system, as measured by maltose hydrolysis, than with fungal alpha-amylase. This observation led to a detailed study of starch saccharification by fungal diastatic preparations with wide variations in the two types of enzyme systems.

Fungal alpha-amylase liquefies the starch and converts it to dextrins and maltose, while the glucogenic enzyme system hydrolyzes maltose, dextrins, and apparently starch itself to glucose. Starch saccharification efficiency showed a higher correlation with glucogenic activity than with alpha-amylase. High glucogenic activity was associated with rapid and almost quantitative glucose formation from starch. Starch degradation with a *Rhizopus* culture filtrate having only a trace of alpha-amylase activity showed production of considerable glucose but no maltose, although the blue-starch iodine reaction persisted.

Potent mold culture filtrates have low Lintner values, for they apparently contain little or no beta-amylase. Nevertheless, they saccharified starch to produce ultimate yields of fermentable sugars that were as high or higher than was obtained by saccharification with the distillers' malt sample used for reference.

In China and Japan starch saccharification by mold enzymes has been practiced for centuries. The Amylo process described by Grove (3) for commercial production of alcohol employed amylolytic fungi

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originating in those countries and was first applied in France. In this process the mold is grown directly in the grain mash which is saccharified by the fungal enzymes thus developed. Erb and Hildebrandt (2) and Underkoefler *et al.* (11, 12) in this country have reported improved alcohol yields when fungal amylases were used commercially to saccharify grain mashes.

Le Mense, Corman, *et al.* (4) at this laboratory have recently reported a method for producing fungal amylases by submerged cultivation of fungi in distillers' thin stillage supplemented with 1% corn and 0.5% calcium carbonate. After 66 hours aeration, the culture liquors were used successfully by Le Mense, Sohns, and co-workers (5) in the pilot plant to replace completely the barley malt conventionally used in alcohol production. These culture filtrates have been used also by Efron and Blom (1) to make saccharine syrups from grain.

At first it was assumed that the major factor contributing to efficient starch conversion by fungal amylase preparations was alpha-amylase activity. Therefore, in a preliminary survey, mold strains were selected on the basis of their ability to produce high levels of alpha-amylase during submerged culture. However, it was soon found that the yields of alcohol on fermentation did not correlate with the alpha-amylase content of the culture filtrates. Subsequent studies indicated that rapid and complete saccharification might be related to the presence of a supplementary carbohydrazase system which was measured by maltose hydrolysis. This "maltase" enzyme is subsequently referred to in this paper as "glucogenic activity." The enzyme or enzyme system involved appears to be capable also of attacking higher glucose polymers such as dextrins and even starch.³

Recognizing the presence of both alpha-amylase activity and glucogenic activity in our fungal amylase preparations, we investigated the correlation between levels of each type of enzyme activity and alcohol yields obtained from corn mashes saccharified with our mold culture filtrates. We also studied the progress of starch hydrolysis by fungal preparations varying widely in each type of enzyme activity and determined the nature of the starch degradation products obtained. Similar studies were made with an extract of distillers' malt for comparative purposes.

Experimental

Fungal amylase preparations were produced by submerged growth of molds in distillers' thin stillage supplemented with corn and calcium carbonate as described by Le Mense, Corman, *et al.* (4).

³ In other papers on this subject from this laboratory (4, 5) and elsewhere the terms "maltase" and "maltase activity" are used. However, objection can be raised to these terms since the enzyme or enzyme system hydrolyzes higher glucose polymers as well as maltose. Accordingly in this paper, we have adopted the new term "glucogenic activity." These different terms in various papers refer, therefore, to the same thing.

Alcohol yields were determined by fermentation tests as follows: A slurry of 50 g. of ground corn and 200 ml. tap water in a 500-ml. Erlenmeyer flask was steamed at atmospheric pressure in the autoclave for 10 minutes. The hot mash was stirred well, cooked at 25 pounds steam pressure for 30 minutes, and then cooled to 72°C. Its temperature was further lowered to 55°-58°C. by vigorously stirring into it a mixture of 25 ml. of culture filtrate and 25 ml. tap water. The flask was placed in a 55°C. water bath for 30 minutes after which the mash was cooled to 30°C. and inoculated with 10 ml. of a 24-hour culture of *Saccharomyces cerevisiae* NRRL Y-567. After 72 hours' fermentation at 30°C. the entire mash was distilled until about 99 ml. was collected in a 100-ml. volumetric flask. The solution was diluted to volume with water and the alcohol yield determined with a dipping refractometer.

Alpha-amylase was determined by the Sandstedt, Kneen, and Blish procedure (8) as modified by Olson, Evans, and Dickson (6, 7).

The amount of alpha-amylase represented by one unit dextrinizes 1 g. of starch (pretreated with excess beta-amylase) in one hour at 20°C.

Glucogenic activity was determined by measuring the extent of conversion of maltose monohydrate to glucose when a reaction mixture containing two volumes of 1.05% maltose monohydrate and one volume of culture filtrate was incubated for two hours at 30°C. A pH of 4.6 was maintained in the reaction mixture by use of 0.137 M acetic acid-sodium acetate buffer. Glucogenic activity is expressed as the percentage of maltose monohydrate hydrolyzed to glucose under these conditions. The extent of hydrolysis was determined by the Somogyi (10) micro sugar method using the 20-minute heating time.

Progress of starch hydrolysis was followed by measuring the development of reducing power at various time intervals by the above-mentioned Somogyi method. Analysis for glucose, maltose, and dextrans in the presence of each other was made by the Somogyi (9) differential yeast fermentation procedure.

Alcohol Production. Alcohol yields obtained by use of some fungal amylase preparations for preliminary saccharification of the corn mashes are given in Table I. It is apparent that alcohol yields correlate more closely with glucogenic activity than with alpha-amylase activity. A high alcohol yield was obtained from the corn mash converted with the culture filtrate from *Aspergillus niger* NRRL 605 in spite of its unusually low alpha-amylase value. High glucogenic activity, however, was present in this preparation. On the other hand, saccharification with a culture filtrate of *Aspergillus foetidus* NRRL 341 with relatively good alpha-amylase value but low glucogenic activity was followed by poor fermentation.

The glucogenic activities of some fungal amylase preparations prepared from the same stock slant under essentially identical conditions sometimes vary. In Table I are listed two such preparations. These were inoculated on different days from the same stock slant of *Aspergillus oryzae* NRRL 464 to standard amylase producing media, and cultivated as usual. Although the alpha-amylase values of both preparations were approximately the same, glucogenic activities differed considerably. The higher alcohol yield was again associated with the preparation of high glucogenic activity, whereas a lower alcohol yield

TABLE I
ALCOHOL YIELDS FROM CORN MASHES SACCHARIFIED WITH FUNGAL AMYLASES

Culture	Glucogenic activity, % maltose hydrolyzed	Alpha-amylase, units per ml.	Ethanol, ¹ proof gal. per bu.
<i>A. niger</i> 330	77.9	5.3	5.24
<i>A. phoenicis</i> 363	69.6	9.7	5.14
<i>A. wentii</i> 378	65.6	2.4	5.14
<i>A. oryzae</i> 464 ²	64.6	3.0	5.40
<i>A. niger</i> 605	61.7	0.1	5.05
<i>A. niger</i> 326	60.6	4.5	5.29
<i>A. niger</i> 354	47.6	3.7	4.97
<i>A. wentii</i> 382	28.5	1.5	4.97
<i>A. wentii</i> 377	25.4	0.9	4.44
<i>A. niger</i> 337	21.9	12.5	5.09
<i>R. delamarei</i> 1705	19.6	trace ³	4.58
<i>R. sp.</i> "Boulard" 1891	19.5	trace ³	4.47
<i>A. oryzae</i> 694	12.2	8.3	4.85
<i>A. oryzae</i> 464 ²	11.5	2.9	4.71
<i>A. foetidus</i> 341	11.4	7.7	4.58

¹ Alcohol yield of 10% malt control was 5.1 proof gallons per bushel.

² Both culture filtrates inoculated from same stock slant.

³ Culture filtrate assayed less than 0.08 alpha-amylase unit per ml.

resulted when fermentation followed the use of the preparation of low glucogenic activity.

Thus it appears that the supplementary carbohydrase system measured by maltose hydrolysis is an important factor in determining the efficiency of starch hydrolyzing fungal amylase preparations used for alcohol production.

Alpha-amylase, on the other hand, appears to be the main source of liquefying action. Conversion by culture filtrates of high alpha-amylase potencies resulted in very thin liquid mashes, while conversion with preparations of lower alpha-amylase activity produced smooth mashes which were not quite as liquid. Conversion with *Rhizopus* and *Mucor* culture filtrates invariably resulted in thick, gummy mashes, apparently because of the lack of alpha-amylase in these preparations.

Progress of Starch Hydrolysis by Mold Enzymes. In view of the common use of total reducing power determinations as a measure of starch saccharifying efficiency, the progress of starch hydrolysis was followed in terms of development of reducing power. Culture filtrates from four mold strains, as well as an extract of 180° Lintner barley malt, were used to saccharify 2% soluble starch. The reaction mix-

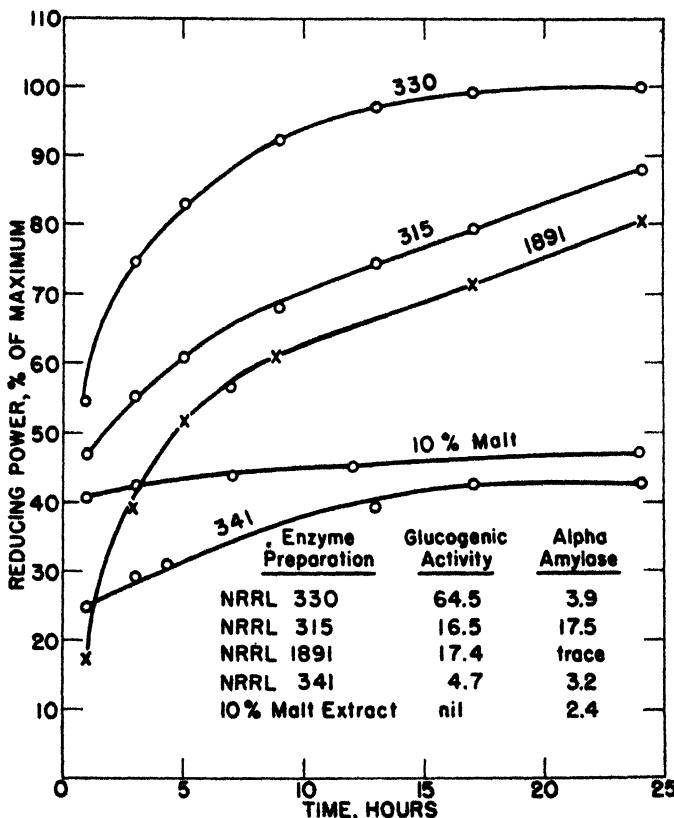


Fig. 1. Starch hydrolysis with various amyloytic preparations at 55°C., in terms of development of reducing power.

tures, consisting of 40 ml. of 2% soluble starch and 4 ml. of culture filtrate, or malt extract, were incubated at 55°C. and pH 4.6. "Maximum" reducing power was that developed by acid hydrolysis on a separate starch aliquot and thus corresponds approximately to the value that would be obtained if the starch were completely hydrolyzed to glucose.

Both the rate and extent of reducing power development increased as the glucogenic activities of the mold culture filtrates increased from

4.7 to 64.5, as shown in Fig. 1. No such relationship exists between fungal alpha-amylase activity and reducing power. These observations substantiate our conclusions from the fermentation studies that fungal alpha-amylase alone is not the determining factor in efficient

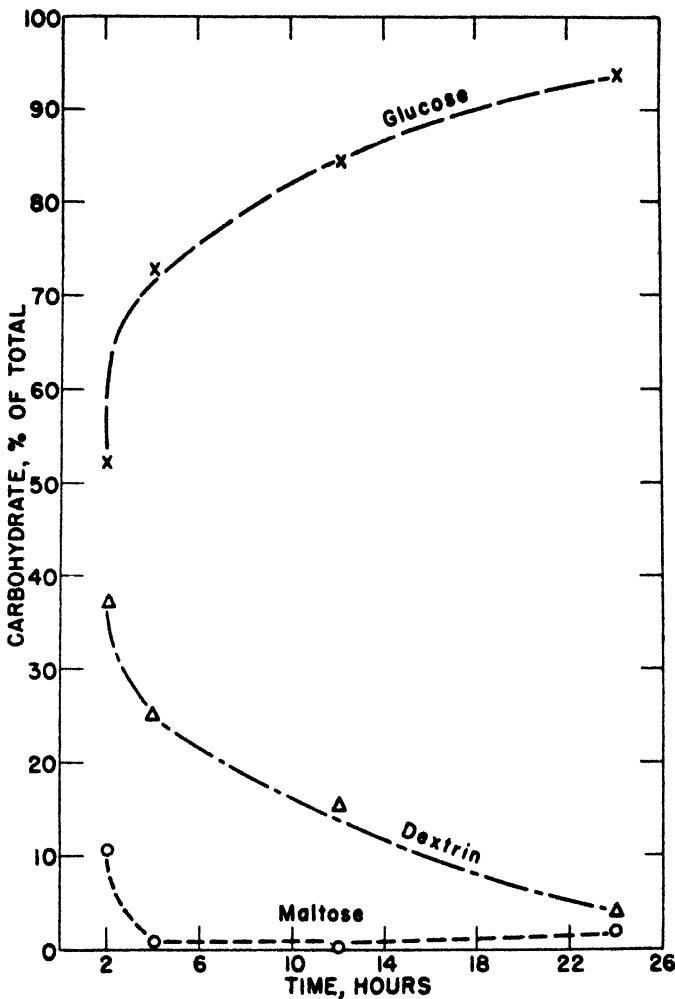


Fig. 2. Starch hydrolysis with *Aspergillus niger* NRRL 330 (at 55°C.) culture filtrate having 64.5 glucogenic activity and 3.9 alpha-amylase units per ml.

starch saccharification, but that a supplementary carbohydrase reflected by high glucogenic activity is also apparently necessary.

The difference in the rate of starch saccharification by mold enzymes from that by malt extract is also demonstrated in Fig. 1. In the case of malt hydrolysis, the reducing power attained at first

analysis (1 hour) was already essentially that ultimately attained at 24 hours, while in the case of mold hydrolysis, reducing power development was much more gradual. For this reason, the Lintner determination (30 minute hydrolysis), so important for malt evaluation, lacks significance when applied to mold culture filtrates. A culture filtrate of *Aspergillus niger* NRRL 330 which had 4.0 alpha-amylase units per ml. and a glucogenic activity of 56.7, when used instead of 5% malt infusion, was found to have a Lintner value of only 34° compared to 180° for the malt. Yet, 5.36 proof gallons of alcohol per bushel of

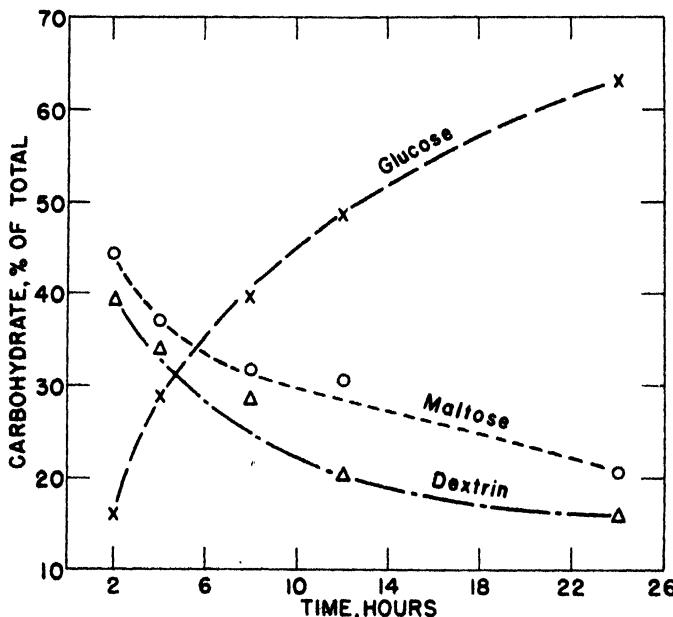


Fig. 3. Starch hydrolysis with *Aspergillus alliaceus* NRRL 315 (at 55°C.) culture filtrate having 16.5 glucogenic activity and 17.5 alpha-amylase units per ml.

corn was obtained through use of this filtrate compared to 5.10 for the malt.

These observations, indicating apparent quantitative production of glucose from starch by potent mold culture filtrates, led to the more detailed hydrolysis investigations that follow.

Composition of Starch Hydrolyzates. The final reducing power developed by most of the mold culture filtrates in the preceding experiments was higher than that developed by malt. Since the latter is known to produce mainly maltose, this observation suggested that substantial quantities of glucose as well as maltose were being produced during starch hydrolysis by molds, and that the total reducing power alone would not be a true index of the amount of fermentable sugars

formed. Therefore, the distribution of glucose, maltose, and unfermentable dextrans as they appeared during hydrolysis of the starch was determined. The starch hydrolysis was carried out as already described for the preceding studies, using the same mold culture filtrates and malt extract.

The course of hydrolysis of 2% soluble starch by a culture filtrate of

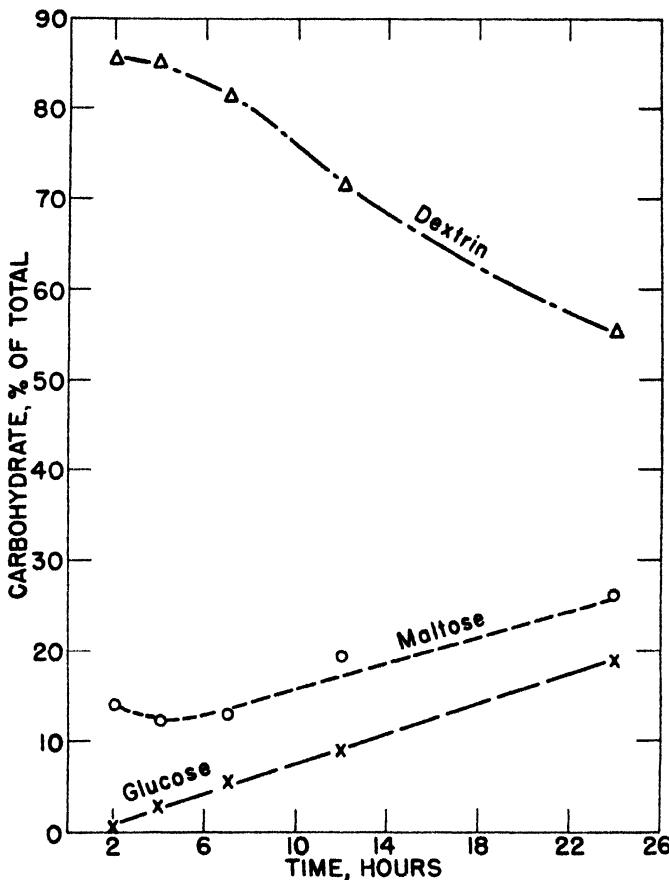


Fig. 4. Starch hydrolysis with *Aspergillus foetidus* NRRL 341 (at 55°C.) culture filtrate having 4.7 glucogenic activity and 3.2 alpha-amylase units per ml.

Aspergillus niger NRRL 330 is presented in Fig. 2. This filtrate had an intermediate alpha-amylase content, high glucogenic activity, and caused rapid development of reducing power (Fig. 1). The hydrolyzate, after 24 hours, was composed of 94% glucose, 2% maltose, and only 4% unfermentable dextrans. The hydrolysis of the starch to glucose was very rapid, over 50% of the hydrolyzate being glucose after 2 hours of incubation, when the first sample was taken.

The culture filtrate from *Aspergillus alliaceus* NRRL 315, which showed only intermediate glucogenic activity but high alpha-amylase potency, gave a lower rate of saccharification (Fig. 3) than did filtrates with high glucogenic activity. The carbohydrates at 24 hours consisted of 63% glucose, 21% maltose, and 16% unfermentable dextrans.

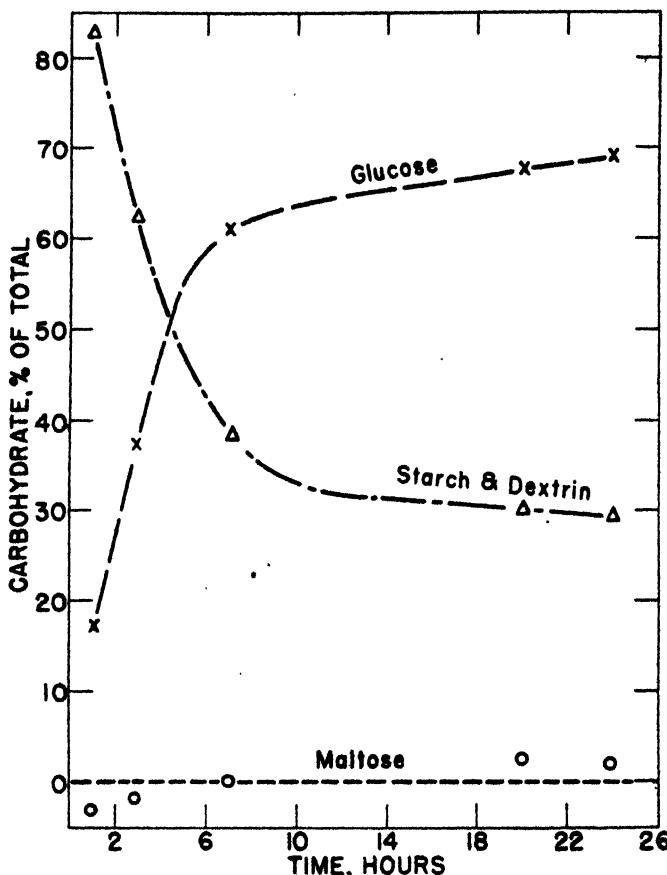


Fig. 5. Starch hydrolysis with *Rhizopus* sp. "Boulard" NRRL 1891 (at 55°C.) culture filtrate having 17.4 glucogenic activity and a trace of alpha-amylase per ml.

It is notable that a high production of maltose occurred in the initial stages of hydrolysis, presumably because of the high alpha-amylase activity of the culture filtrate. The dextrin content as well as the 45% maltose in the 2-hour hydrolyzate gradually decreased as hydrolysis progressed.

The course of starch hydrolysis with the culture filtrate from *Aspergillus foetidus* NRRL 341 is illustrated in Fig. 4. This filtrate

had low glucogenic activity and intermediate alpha-amylase activity. Production of glucose was slow, with about 55% dextrin remaining after 24 hours hydrolysis. From the shape of the curves it appears that the dextrin is gradually degraded and that both maltose and glucose formation followed the dextrinizing reaction.

The culture filtrate from *Rhizopus* sp. "Boulard" NRRL 1891 (the

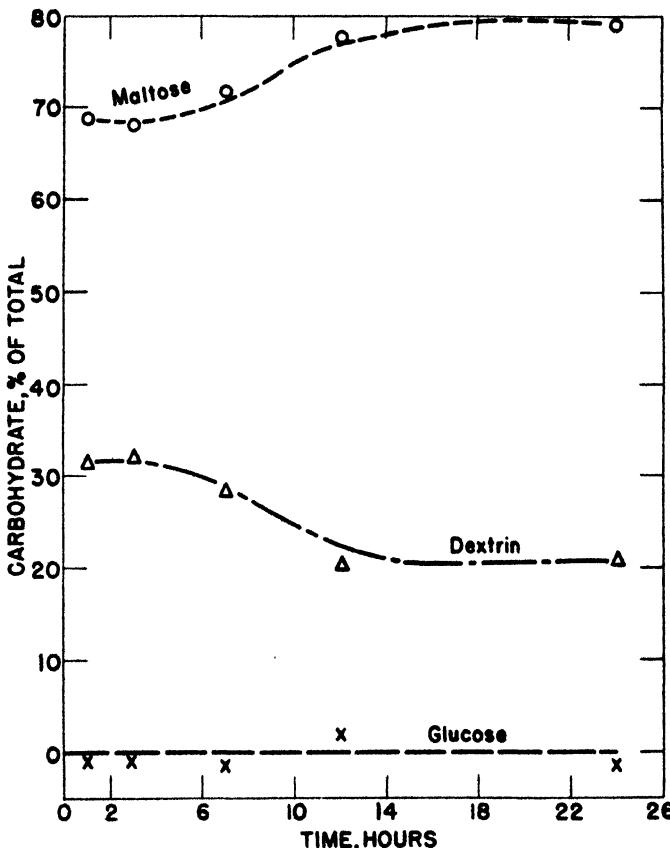


Fig. 6. Starch hydrolysis with 10% extract of 180° Lintner malt (at 55°C.) that assayed 24.0 alpha-amylase units per gram.

strain used in the Amylo process for alcohol production) was of particular interest. This filtrate contained intermediate glucogenic activity but almost no alpha-amylase activity. It caused fairly rapid liberation of glucose with no apparent production of maltose, as shown in Fig. 5. The blue starch-iodine reaction persisted for more than 24 hours, indicating that the dextrin value also included starch which had been hydrolyzed slightly, if at all. At 24 hours the hydro-

lyzate contained 70% glucose, 30% dextrin and starch, and, within experimental error, no maltose. The glucogenic enzyme system in this instance apparently attacked the starch directly to produce glucose.

For comparative purposes, the results of hydrolyzing starch with a 10% extract of barley malt are given in Fig. 6. Malt caused the production mainly of maltose with no glucose, and left 21% unfermentable dextrans in the 24-hour hydrolyzate. The absence of glucose confirmed our finding that no glucogenic activity was present. It should be noted that most of the hydrolysis was completed in the first two hours, when 68% of the starch had been converted to maltose.

Discussion

These experiments indicate that the diastatic system of some mold culture filtrates is primarily composed of a liquefying-dextrinizing enzyme and a saccharifying enzyme system. Thus starch hydrolysis by these fungal preparations is a dual type of saccharification process similar to hydrolysis by malt. Although the action of fungal alpha-amylase is similar to that of malt alpha-amylase, the saccharifying glucogenic enzyme system which liberates glucose differs markedly from saccharifying malt beta-amylase which produces maltose.

In fungal enzyme preparations, alpha-amylase serves to liquefy the starch paste and to rupture the starch molecule with the production of dextrans and, eventually, a limited quantity of maltose. This follows from the fact that fungal preparations with appreciable alpha-amylase activity but of low glucogenic potency caused accumulation of maltose with degradation of dextrin and, in a negative sense, from the fact that no maltose was formed by the *Rhizopus* filtrate in which only a trace of alpha-amylase was present.

The glucogenic enzyme system appears to be active both upon maltose and upon more complex polymers of glucose, since the *Rhizopus* preparation which contains only a trace of alpha-amylase was capable of appreciable degradation of starch to glucose without loss of the blue iodine color. Furthermore, in those cases where a high glucogenic activity is present such as *Aspergillus niger* NRRL 330 (Fig. 2), starch hydrolysis proceeds very rapidly to glucose.

Fungal alpha-amylase and the glucogenic enzyme system apparently supplement each other in the production of fermentable sugars during starch hydrolysis. Thus a comparison of Figs. 3 and 5, in which starch hydrolysis was due to preparations of similar glucogenic activities, shows that there was half as much residual dextrin in the 24-hour hydrolyzate from *Aspergillus alliaceus* NRRL 315, presumably due to the supplementary action of its high alpha-amylase com-

ponent. In Table I the greater alcohol yield associated with *Aspergillus niger* NRRL 337 compared with *Aspergillus wentii* NRRL 377 is also indicative of this supplementary action.

In view of the importance of beta-amylase in the malt diastatic system, its possible presence in the mold enzyme system is of considerable interest. While our information on this subject is as yet quite limited, the low Lintner values of potent fungal preparations indicate the absence of any appreciable beta-amylase activity in mold culture filtrates.

These experiments demonstrate that the evaluation of fungal preparations should include measurement of glucogenic activity as well as alpha-amylase activity, because the rate and completeness of starch hydrolysis depend to a large extent on the activity of the carbonhydrase enzyme system that can be measured by its action on maltose.

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EVALUATING THE NUTRITIVE VALUES OF SEVERAL BREADS BY GESTATION-LACTATION PERFORMANCE¹

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ABSTRACT

Six types of bread which had previously been evaluated nutritionally by the rat-growth method were re-evaluated by using the gestation-lactation period as the test period. The females were fed the breads supplemented with 0.1 ml. U.S.P. cod-liver oil and following the growth period they were fed a further addition of 0.1 mg. of alpha tocopherol. Three litters were produced on the diets and statistical analysis revealed that the performance of the third litter gave the most reliable data for evaluation.

Nonfat milk bread was nutritionally superior to water bread, whole wheat bread, and whole wheat nonfat milk bread as determined by statistical analysis.

The differences in the results between enriched water bread and water bread were not statistically significant.

Whole wheat bread was decidedly inferior to all other types of bread with the exception of whole wheat nonfat milk bread which was not significantly superior to it.

The nutritive values measured by gestation-lactation performance were not similar to those obtained by growth studies. The former is a more critical period nutritionally than the growth period, and its use is a more effective means of evaluating diets. In evaluating diets or supplements the period in the life cycle used for testing should be indicated, and differences obtained in one period of the life cycle should not be assumed as applying to another period.

In evaluating the over-all nutritive value of diets or in assaying the effects of single nutritive adjuvants to basal diets, the short time rat-growth method has been extensively employed. If some other period in the life cycle of the rat were used, the results obtained might not be similar to those obtained during growth. Moreover, experimental work in other periods might indicate a period that was even superior to the growth period in ascertaining the comparative nutritive values between diets or supplements under investigation. The literature offers some support to the speculation that the gestation-lactation period might be more critical nutritionally than the growth period. An opportunity presented itself to work with female rats that had been used in a growth study, starting when they weighed approximately 40 g. and continuing for 8 weeks. These females were continued on the same diets and were on test during three successive gestation-lactation periods.

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In the rat-growth studies grams of gain or grams of food required for a gram of gain have been taken, most generally, alone or in combination, to measure the differences obtained on one or more diets. At times, these have been supplemented by carcass analysis, special determinations, such as hemoglobin and ash content of the bones, and in some instances differences in palatability have been ruled out by equalizing the food intake or by feeding for equal gains. A rather extensive bibliography is presented to support the contention that the gestation-lactation period has possibilities for measuring over-all nutritional effects. It may be a critical period nutritionally, and it affords several observations that lend themselves to measurements. In gestation-lactation there are for observation conception or failure to conceive, the course of pregnancy, gain or loss in weight of the female during pregnancy, the number and individual weights of the young at birth, the number still-born or alive, mortality of the young during lactation which may be recorded in time intervals, the gain or loss in weight of dam, and the number and individual weights of the animals weaned.

In swine nutrition literature there are several papers reporting the inadequacies of experimental rations for normal gestation and lactation. These have been reviewed by Krider, Fairbanks, and Carroll (15) and Fairbanks, Krider, and Carroll (6). Pastures reduced pig mortality in the investigation of Aubel, Hughes, and Leinhardt (2) and Hogan and Johnson (13). Asdell and Willman (1) report the mortality of pigs was twice as high in spring farrowings as in fall farrowings, and it is logical to assume that nutrition during gestation was exerting its influence along with differences in seasons. The number of pigs farrowed and their weights and strength at birth were used as measures of nutritional adequacy of rations during gestation by Gardner (10) and Freeman (9). Some differences noted in gestation-lactation studies are illustrated by the work of Hogan (12) in which 81% of the pigs were weaned on one feeding regime as compared to 51% on another. Hogan and Johnson (14) showed that mortality of young pigs could be reduced by improved nutrition and report that 76% of the pigs farrowed were weaned on a ration that had been quite unsatisfactory before supplementation. Fairbanks, Krider, and Carroll (6) report pig mortalities ranging from 5% to 93% which they attributed to differences in the nutritive value of the gestation-lactation rations. The percentages of pigs weaned varied from 7% to 83% due to differences in the levels of nutrition during gestation and lactation.

Hart, McCollum, Steenbock, and Humphrey (11) indicated that the rations fed pregnant heifers profoundly affected the viability and vigor of their calves.

In human nutrition, the paper of Burke, Beal, Kirkwood, and Stuart (3) is of interest. Their work, including 216 cases, showed the influence of the diet of the mother during gestation on the condition of the infants. Those mothers with diets rated as good or excellent delivered babies of which 42% had pediatric ratings of superior and 55% one or two minor defects. These are to be contrasted with the babies from mothers receiving diets during gestation which were rated as poor or very poor. Only 2.5% of the babies were excellent, 2.5% had one minor defect, 2.8% were in fair or poor condition, while 67% were considered to be in very poor condition. Burke, Harding, and Stuart (4) found a significant relationship between the protein content of the gestation diet and the birth length, birth weight, and the pediatric ratings of the babies.

Wright and Haag (23) working with rats found that l-cystine promoted lactation when added to diets in which the proteins were furnished by alfalfa leaf meal. Normal reproduction and improved lactation in rats were obtained by supplementing rations that had failed to support normal reproduction and lactation, according to the reports of Ross (18) and Cunha (5). The diets fed to breeding females influenced the incidence of congenital abnormalities in their offspring in the investigations of Warkany, Nelson, and Schraffenberger (22). The most recent paper is that of Spitzer and Phillips (21). On their basal diet, more than 35% of the female rats failed to conceive, resorption and toxemia frequently occurred, and the mortality was high one or two days following parturition. Certain supplementations to the basal ration improved the gestation-lactation results.

Materials and Methods

The Bread Samples. The six different kinds of bread fed during the three gestation-lactation periods were the same as those fed during growth by Riggs, Beaty, and Johnson (17). The bread samples were prepared in the National Dairy Research Laboratories, Baltimore, Maryland, as required. Since the experiment lasted several months it seemed advisable to prepare fresh samples periodically. However, all bread samples were prepared from the same lots of white flour and of whole wheat flour representing the whole wheat berry. The bread formulae used throughout were typical of those formulae employed by commercial bakers in the eastern section of the United States, being described as (1) sponge: 58% flour, 2% yeast, 0.5% bread improver, and 41% water; (2) dough: 42% flour, 4% sugar, 2% salt, 2% malt, 1.5% shortening (hydrogenated vegetable oil), and 27.7% water. The supplements were added at the time the dough was made.

The following types of bread were studied:

- 1.G. *Water bread*. White flour without supplement.
- 2.G. *Enriched water bread*. White flour enriched with thiamine, riboflavin, niacin, and iron.
- 3.G. *Nonfat milk bread*. 6% nonfat dry milk solids based upon the weight of white flour.
- 4.G. *Enriched nonfat milk bread*. Enriched as in 2G and nonfat milk solids as in 3G.
- 5.G. *Whole wheat bread*. Whole wheat flour, representing the whole wheat berry.
- 6.G. *Whole wheat nonfat milk bread*. 6% nonfat dry milk solids based upon the weight of whole wheat flour.

The breads were analyzed for the usual constituents by standard methods of analysis and assayed for the vitamins by recognized chemical and microbiological methods. The attempt was made to enrich the white flour to 10% above the minimum levels for thiamine, riboflavin, niacin, and iron as specified in the new standards for enriched flour (Federal Register, 7). The composition of the breads, Table I, indicates that the vitamin enrichment ingredients are above the minimum requirements for enriched bread as specified in the Federal Register (8) with the exception of thiamine, which assayed 0.92 mg. per pound rather than 1.1 mg. as prescribed.

The Experimental Animals. Females of the Sprague-Dawley strain, weighing from 35 to 50 g., were fed ad libitum the same bread diets as reported by Riggs, Beaty, and Johnson (17) and with essentially the same growth response as indicated by the following results at the end of a growth period of 8 weeks. The average final weights of rats receiving the six bread diets were: water bread, 68.6 g.; enriched water bread, 76.9 g.; nonfat milk bread, 128.0 g.; enriched nonfat milk bread, 111.0 g.; whole wheat bread, 104.4 g.; and whole wheat nonfat milk bread, 153.4 g. The corresponding figures for grams of bread per gram gain were water bread, 10.36 g.; enriched water bread, 8.82 g.; nonfat milk bread, 5.10 g.; enriched nonfat milk bread, 5.41 g.; whole wheat bread, 6.03 g.; and whole wheat nonfat milk bread, 4.52 g. During the growth period, bread, water, and a daily dose of 0.1 ml. U.S.P. cod-liver oil were the only constituents of the diet. Eight female rats of each group were continued for four more weeks before mating, each group receiving its respective diet as previously fed with the addition of 0.1 mg. of alpha tocopherol per rat per day.

Following the 12 weeks' feeding the females were placed in group cages, four females to the cage, and one male rat from the stock colony was introduced into each cage. The males were rotated at the

end of each week so that no one male rat remained with any four females for more than one week. Sufficient males were available so that one group of males could usually be kept on the stock diet for one week and then in the mating cages on the bread diets the following week. The rotating of the males among the breeding cages and the

TABLE I
COMPOSITION OF DRY BREAD CRUMBS

Bread	Description of bread	Mois-ture	Crude protein	Ether extract	Crude fibre	Ash	Thia-mine	Ribo-flavin	Niacin
1G	White water bread	9.4	12.9	3.1	0.4	2.68	0.29	0.54	5.93
2G	White water bread, enriched	9.4	13.0	3.0	0.5	2.70	1.34	1.36	17.42
3G	White water bread plus 6% nonfat dry milk solids	8.7	13.9	2.8	0.5	2.93	0.29	0.90	5.50
4G	White water bread, enriched plus 6% nonfat dry milk solids	8.4	13.9	3.0	0.4	2.81	1.36	1.84	20.99
5G	100% whole wheat bread	10.1	14.5	2.6	1.8	3.19	1.17	0.98	15.53
6G	100% whole wheat bread plus 6% nonfat dry milk solids	9.6	15.6	2.9	1.7	3.35	1.04	1.25	14.83

Chemical composition of bread (38% moisture) calculated from above analyses

1G	White water bread	38.0	8.8	2.1	0.3	1.83	0.20	0.37	4.06
2G	White water bread, enriched	38.0	8.9	2.1	0.4	1.85	0.92	0.93	11.92
3G	White water bread plus 6% nonfat dry milk solids	38.0	9.5	1.9	0.3	1.99	0.20	0.61	3.74
4G	White water bread, enriched plus 6% nonfat dry milk solids	38.0	9.4	2.0	0.2	1.90	0.92	1.24	14.20
5G	100% whole wheat bread	38.0	10.0	1.8	1.3	2.20	0.81	0.68	10.72
6G	100% whole wheat bread plus 6% nonfat dry milk solids	38.0	10.7	2.0	1.2	2.30	0.71	0.86	10.18

feeding of the males on the stock diets during alternate weeks were done to reduce to the minimum the influence of variations among males and their nutritional status on the performance of the females during gestation and lactation. The same male rats were used during the three gestation-lactation periods.

The females were weighed three times each week and observed for pregnancy. When the female was obviously pregnant she was placed in an individual cage where she remained until weaning her litter at 25 days following parturition.

When all of the females fed the six breads had completed the first gestation-lactation period, they were returned to the mating cages and

the males were again introduced. The length of time between either the weaning or destruction (death or cannibalism) of the first litter and the time the females were returned to the mating cages depended upon the time of birth of the first litter. The rest period was longer in the case of females on diets of nonfat milk bread and whole wheat bread than for those on diets of water bread and enriched water bread.

Following the weaning or destruction (by death or cannibalism) of the second litter, the females were immediately returned to the mating cages without any rest period. The technique employed for the third litter is preferred for test purposes.

Results

The results during gestation and lactation with the first and third litters only are reported in Table II. The second litters have been omitted, but references will be made to results of the second litter when they have a contribution to make.

Statistical Analysis. The data for the number of rats in each litter, the average birth weight of rats in each litter, and the percentage of

TABLE II
RESULTS DURING GESTATION AND LACTATION
(Ad libitum feeding)

Items compared	Group numbers and diets									
	1G		2G		3G		4G		5G	
	Water bread	Enriched water bread	Nonfat milk bread	Nonfat milk bread	Enriched nonfat milk bread	Whole wheat bread	Whole wheat bread	Whole wheat nonfat milk bread	Whole wheat nonfat milk bread	Whole wheat nonfat milk bread
GESTATION										
Number of litters born	7 ¹	8	8	5	8	8	8	8 ²	8	8
Number of rats born	50	38	45	33	51	62	53	49	38	70
Avg. no. rats per litter	7.1	4.8	5.6	6.6	6.4	7.8	6.6	6.1	8.0	8.8
Avg. birth wt. grams	5.0	5.3	5.0	5.6	4.8	5.6	5.6	5.7	5.5	5.8
LACTATION										
Avg. no. rats weaned per litter	0.9	1.0	0.5	2.4	2.4	4.3	2.8	3.0	1.0	0
Avg. weaning wt. (grams)	26.6	18.4	12.5	18.9	18.6	23.0	20.0	28.7	23.0	0
Percentage of rats died:										
Birth to 3rd day	58.0	34.2	46.7	39.4	43.1	21.0	30.2	14.3	55.6	72.0
3rd Day to 7th day	30.0	28.9	26.7	21.2	17.6	17.7	7.5	16.3	5.6	24.0
7th Day to 14th day	0	15.8	11.1	3.0	2.0	6.5	9.4	18.3	8.3	4.0
14th Day to 25th day	0	0	6.7	0	0	0	11.3	4.1	8.3	0
Percentage of rats weaned	12.0	21.1	8.9	36.4	37.3	54.8	41.5	49.0	22.2	0

¹ One female failed to conceive but produced two subsequent litters.

² Two females failed to conceive at any time.

rats in each litter reaching the weaning stage have been submitted to statistical analysis. The first two series of data (rats per litter and average birth weights) yield arithmetic averages to which the analysis of variance could be applied as a test of significance. The third series (percentage of rats weaned), being a set of percentage figures, is more properly analyzed by the chi square test, a technique appropriate to percentages.

The chi square values indicated that the data were not homogeneous by litters, except in the case of water bread and possibly with enriched nonfat milk bread. The number of significant differences between the 15 possible combinations between breads taken two at a time increased from the first to the third litter, indicating an accumulative effect in nutritive value of the six breads.

The statistical study revealed that the greatest number of differences proved to be significant in the third litters and successively increased from the first to the third litters, reflecting the longer time the mothers of the third litters had been on their respective feedings and hence a more extended opportunity for the differences in breads to demonstrate themselves. The data for the third litter are therefore taken as the most significant data in appraising the over-all nutritive value of the six breads under consideration.

Average Number of Rats Born per Litter. The number of live rats born per litter may be an indication of the nutritive value of the diets to permit ovulation, conception, full-term gestation, and parturition beyond the extent that these performances are controlled genetically. In the third litters (Table II), the differences between the water breads and all other breads are significant by analysis of variance in only three cases, nonfat milk bread ($F = 9.89$, df 1 and 14), whole wheat bread ($F = 12.61$, df 1 and 14), and whole wheat nonfat milk bread ($F = 16.25$, df 1 and 14). This analysis of the differences most likely to be significant, namely between water bread and all other breads, indicates that it is unlikely that statistically significant differences can be established by the other types of bread or by order of litters.

In the planning of this preliminary long-time experiment covering three gestation-lactation periods it was decided not to equalize the litters at birth, as the size of the litters and the ability of the females to wean the pups born were considered a part of the data desired. In future and more refined experiments designed to explain differences reported in this trial, it might be advisable to consider equalization of litters.

Average Birth Weights. It is difficult to correlate the average birth weight with the nutritional adequacy of the diet since this figure is influenced by the number of rats born. The results of the first and

third litters, expressed in grams, are enumerated in Table II. By the analysis of variance the differences in the third litter between water bread and the other breads are not significant. In fact an analysis between the differences most likely to be significant, namely water bread and the other breads, indicates only one significant difference of the 15 comparisons and that was between water bread and whole wheat nonfat milk bread in the first litter ($F = 7.47$, df 1 and 14). It is unlikely that any other statistical difference can be established by types of bread and order of litters.

Percentage of Rats Weaned. The most significant data for evaluating the over-all nutritive values of the six breads fed seem to be in the data of percentage of rats weaned in each litter (see statistical analysis) of the third gestation-lactation period (Table II). The total percentage of rats weaned for the six breads are: nonfat milk bread, 54.8; enriched nonfat milk bread, 49.0; enriched water bread, 36.4; water bread, 21.1; whole wheat nonfat milk bread, 7.1; and whole wheat bread, 0.

The females on the water bread diet weaned a significantly lower percentage of rats than the females receiving nonfat milk bread ($P \leq 0.01$) and enriched nonfat milk bread ($P \leq 0.01$) and a significantly higher percentage of rats than those receiving whole wheat bread ($P \leq 0.01$) and whole wheat nonfat milk bread ($P \geq 0.02$).

The females fed enriched water bread weaned a significantly higher percentage of rats than the females receiving whole wheat bread ($P \leq 0.01$) and whole wheat nonfat milk bread ($P \leq 0.01$).

The females receiving nonfat milk bread weaned a significantly higher percentage of rats than those fed water bread ($P \leq 0.01$), whole wheat bread ($P \leq 0.01$), and whole wheat nonfat milk bread ($P \leq 0.01$).

The females on the enriched nonfat milk bread weaned a significantly higher percentage of rats than those on water bread ($P \leq 0.01$), whole wheat bread ($P \leq 0.01$), and whole wheat nonfat milk bread ($P \leq 0.01$).

The females on whole wheat bread weaned a significantly lower percentage of rats than those on all other breads with the exception of whole wheat nonfat milk bread in which the difference was statistically insignificant.

All other comparisons were not statistically significant.

At the time of mating for the first litter there was an extreme difference of 90 g. in the average weight of the rats (water bread, 95 g.; whole wheat nonfat milk bread, 185 g.). This extreme difference had been reduced to 47 g. when the females were mated for the third litter (water bread, 173 g.; whole wheat nonfat milk bread, 220 g.). Body size as

indicated by body weight might be expected to influence performance during gestation-lactation. Yet, the smaller rats on white water bread were able to wean a significantly larger number of young of the third litter than the larger females receiving whole wheat nonfat milk bread or whole wheat bread (184 g.). This accomplishment of the rats on water bread is further emphasized by the fact that they produced significantly smaller litters than those receiving whole wheat nonfat milk bread and whole wheat bread.

Discussion

Riggs, Beaty, and Johnson (17) in a growth study with rats concluded that "the addition of 6% nonfat dry milk solids improves the nutritive value of water bread, enriched water bread, and whole wheat bread." The gestation-lactation results indicate the nutritional superiority of the nonfat milk bread over water bread, whole wheat bread, and whole wheat nonfat milk bread.

The growth studies supported the conclusion that "enrichment at the new levels caused a slight improvement in the nutritive value of water bread which can be observed by paired-feeding for equal gain." The differences between these two breads were not statistically significant in the gestation-lactation studied (ad libitum feeding).

The most surprising results obtained during the gestation-lactation periods were with whole wheat bread and whole wheat nonfat milk bread. Riggs, Beaty, and Johnson (17) concluded from their growth studies, "Water bread supplemented with 6% nonfat dry milk solids and enriched to the levels of the new standards is equivalent in nutritive value to whole wheat bread of average composition as measured by grams of solids required to produce a one-gram gain and the chemical composition of the carcasses of the experimental animals." The results obtained during gestation-lactation clearly indicated that whole wheat bread was decidedly inferior to all other types of bread with the exception of whole wheat nonfat milk bread which was not significantly superior to it.

The results obtained with whole wheat bread during gestation and lactation are deserving of special comment. In the first litters the females weaned only 22.2% of the rats born, and this figure was reduced to 7.5% in the second litter, while the females were not successful in weaning a single rat from the third litters. In both the second and third litters, the females receiving whole wheat bread weaned the lowest percentage of rats. In the second litter, this percentage was significantly lower than enriched water bread ($P \leq 0.01$), enriched nonfat milk bread ($P \leq 0.01$), and whole wheat nonfat milk bread ($P \leq 0.01$). In the third litters, the percentage of rats weaned on the whole wheat

bread diet was significantly lower than any other bread except whole wheat nonfat milk bread in which the difference was not significant. The fertility of the females on the whole wheat breads was not adversely affected.

The experimental technique employed in this initial work is not capable of explaining in terms of nutrition why observed differences occur. At present only the differences are of interest, and further experiments which are better controlled and more refined in procedures will be conducted in an effort to explain the reported observations. Mitchell, Hamilton, and Shields (16) concluded, "There is something in whole wheat, as compared with patent white flour, that impairs calcium utilization." Riggs, Beaty, and Johnson (17) observed that "whole wheat breads vary in nutritive value depending upon the type of whole wheat flour used."

The effect of the addition of nonfat dry milk solids to whole wheat flour on percentage of survivals is noted in the results presented in Table II. The females weaned 46.8% of the pups of the first litter, 48.3% in the second litter, and only 7.1% in the third litter. The addition of nonfat dry milk solids improved the results materially with the first and second litters, but the milk solids, in the amounts used, were not able to maintain a high percentage of pups weaned in the third litter.

These results are not necessarily at variance with the work reported from Sherman's laboratory if differences in the amounts of milk solids fed are considered. Diet A used by Sherman was made up of one-sixth dry whole milk and five-sixths whole wheat plus 2% of sodium chloride based on the weight of the wheat. Diet B consisted of one-third dry whole milk and two-thirds whole wheat plus 2% of sodium chloride based on the weight of the wheat. Sherman and Quinn (20) reported that rats had been maintained for 14 generations on Diet A and for 17 generations on Diet B. The diet containing the higher proportion of dry whole milk produced faster growth and the females reproduced at an earlier age, continued fertile for a longer time, produced larger litters, and raised a greater number of young to weaning age. When the rat colony was in its thirty-fourth generation, Sherman (19) reported that on Diet B, infant mortality had been less and longevity had been increased by 10%. The improvements noted were attributed to the additional amounts of dry whole milk in Diet B.

Mitchell, Hamilton, and Shields (16) stated, "The nutritive deficiencies of patent white flour, and of bread made from it with no nutritive supplements, have been so widely publicized as to need no further comment. However, the conception thus impressed on the public mind that bread is deficient in the essential nutrients would seem

to apply more to the bread formula used many years ago than to modern commercial bread." With these statements in mind it is interesting to note the results obtained with bread made from patent white flour fed continuously during growth and three gestation-lactation periods. The eight females produced 38 rats in the third litter averaging 4.8 rats per litter and an average birth weight of 5.3 g. per rat. The females of this group took much longer to conceive their first litters than all other groups as indicated by an average of 74 days from the time the male was put in the cage until the litter was born. This difference was markedly reduced for the second litter and had disappeared for the third litter. Based on the percentage of rats weaned from the third litters, water bread is nutritionally equal to enriched water bread ($P \geq 0.05$) and significantly superior to both whole wheat bread ($P \leq 0.01$) and whole wheat nonfat milk bread ($P \geq 0.02$).

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STARCH GELATINIZATION STUDIES. II. A METHOD FOR SHOWING THE STAGES IN SWELLING OF STARCH DURING HEATING IN THE AMYLOGRAPH¹

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ABSTRACT

The stages of swelling occurring during gelatinization of certain starches may be shown by viscosity measurements in the amylograph if a viscous water-binding dispersion medium with proper temperature-viscosity characteristics is employed. This fact is demonstrated herein by utilization of sodium alginate and high viscosity type carboxymethyl cellulose. Starches studied include those of corn, wheat, potato, waxy maize, tapioca, and wrinkled pea.

The gelatinization of certain starches in water with heat takes place in definite stages. Katz referred to the stages as first and second order in connection with the gelatinization of wheat starch (4). An indication by volumetric measurements that these stages are related to changes in size of the granules also demonstrated that the size of granules of certain starches increases by steps with increasing temperature (5). Curves quite similar in nature were produced by study of light transmission during heating of some of the same starches (2). The light transmission studies indicated that the first step in the gelatinization of wheat starch occurs between the temperature of 55°C. and 70°C. and that the greatest effects with rising temperature occur in the second step.

The amylograph as used in cereal laboratories has shown indica-

¹ Manuscript received December 1, 1947.

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tions of the effects of varied rates of swelling in different temperature ranges. However, the curves produced have not adequately shown the swelling that occurs at lower temperatures, particularly for those starches where the steps occur at widely separated temperatures.

It has been shown that as the concentration of wheat starch in a suspension gelatinized in the amylograph is raised, the first indications of swelling of starch are apparent at lower temperatures (1). When, for example, 7% wheat starch dispersion is gelatinized in water, no indications of swelling are apparent to approximately 86°C. From 86°C. to a peak at 93°C. the viscosity increases rapidly, thereafter decreasing due to mechanical action. When a 10% wheat starch dispersion is gelatinized, the first indications of swelling are evident at 71°C. The peak viscosity occurs at a slightly lower temperature than for the 7% concentration, but the more rapid decrease in viscosity after the peak is evidence of greater mechanical breakdown of granules. At the 10% concentration, swelling of granules is effectively shown over the range from 71°C. to about 93°C. or a span of 22°C. Any further increase of starch concentration would result in a viscosity greater than that measurable by the machine. Up to a 10% concentration it is not possible to produce strong evidence of two stages of gelatinization for wheat starch if water is used as the dispersion medium. It appears that some modification of the technique other than that of changing levels of wheat starch must be employed if the stages of gelatinization are to be studied by viscosity measurements.

Employment of a viscous dispersion medium in place of water in gelatinization studies should afford several advantages. Primarily, it should magnify the viscosity effect of small changes in size of granules. The low starch concentration required to give evidence of swelling would reduce the friction between granules and consequent mechanical breakdown when the swelling is at a maximum.

The purpose of this article is to demonstrate how the accepted technique of amylograph starch gelatinization studies may be modified to show more adequately the swelling that occurs during heating of some starches.

Materials and Methods

Description, function, and uses of the Brabender Amylograph were published by Anker and Geddes (1) and by Brown and Harrel (3). The essential difference between the technique described herein and that used in other starch viscosity studies with the amylograph is that a viscous, water-binding dispersion medium of low solids content is utilized in place of water.

Dispersions of sodium alginate and carboxymethylcellulose were

made by adding weighed quantities of the powders to warm distilled water while agitated in a Waring Blender. The dispersion was weighed into the amylograph bowl and the temperature held at 40°C. while the machine was operated until the viscosity curve became flat. A weighed amount of starch was added and dispersed as completely as possible with a spatula. The mixture was afterward held at 40°C. and stirred for 5 minutes to disperse the starch further and bring the viscosity to constant value. The temperature was then increased at the rate of 1.5°C. per minute to 100°C.

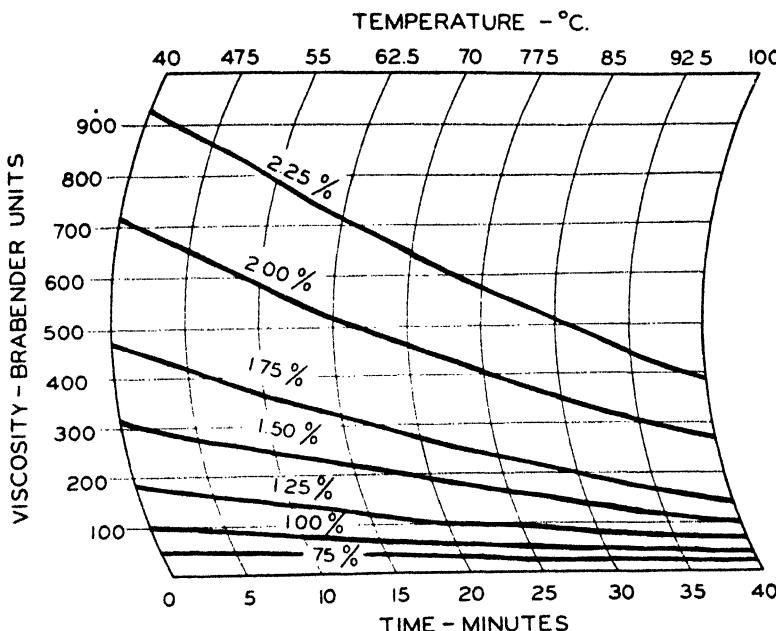


Fig. 1. Relation of viscosity to temperature of various concentrations of sodium alginate from 0.75% to 2.25% solids. Samples of 350 g. were used in all cases.

The lowest concentration used in preparation of curves in this work is 0.75% of sodium alginate; the highest concentration is not known as it is a function of the water taken from the dispersion medium by the starch during gelatinization. It is quite improbable that the effective concentration of sodium alginate in these experiments ever reaches a concentration of 2.25% based on the water not bound by the starch.

Temperature Effect on Viscosity of Dispersion Medium. The effect of temperature on various concentrations of sodium alginate was investigated within the range of concentrations of 0.75% to 2.25% solids; 350 g. of dispersion were used in each case. Fig. 1 shows that

for all concentrations of sodium alginate the viscosity changes at reasonably uniform rates and that in no case is there an increase of viscosity with increase of temperature.

Effect of Concentration of Dispersing Medium. Portions of 25 g. of wheat starch were gelatinized in 350 g. each of water and dispersions of alginate at concentrations of 0.75%, 1.00%, and 1.25% solids.

As is shown by Fig. 2, the contour of the curves resulting from the gelatinization of starch in a viscous-dispersing medium is influenced by the solids content of the dispersing medium. This may reasonably

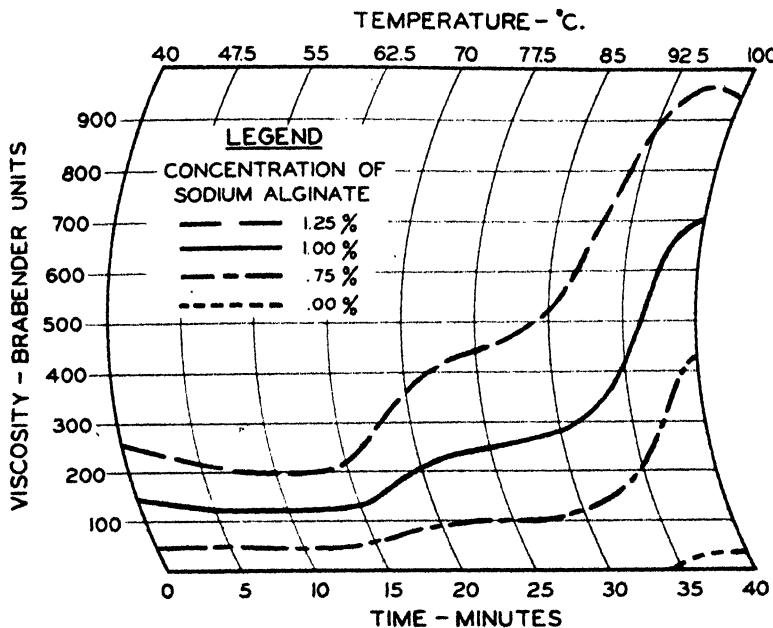


Fig. 2. Effect of the viscosity of the dispersing medium on the contours of the gelatinization curve of wheat starch. Starch samples of 25 g. were dispersed in 350 g. of medium in each case.

be expected because of the complex relations of viscosity to concentration of dispersions.

It will be noted that the first effects of swelling of the starch are indicated at a temperature of 90°C. when the dispersing medium is distilled water only. However, in the starch-alginate-water system the first indications are shown at about 55°C. and change little with wide variations in concentration of sodium alginate.

Effect of Starch Concentration. As the dispersing medium for gelatinization of various levels of wheat starch 350 g. of 1.25% sodium alginate dispersion were used. The levels were 0, 12.5, 25, and 50 g. of wheat starch.

It is shown in Fig. 3 that as the level of starch gelatinized in a given dispersion medium is increased, the temperature of initial swelling will be more sharply defined. It is shown, also, that the temperature of initial indication of swelling remains fairly constant as starch levels vary in contrast to the situation when water alone is used as the dispersing medium (1).

Varied levels of starch produce greater effects in contours of the curves than does variation of concentration of the medium, as may be seen by comparison of Figs. 2 and 3. While one curve produced by

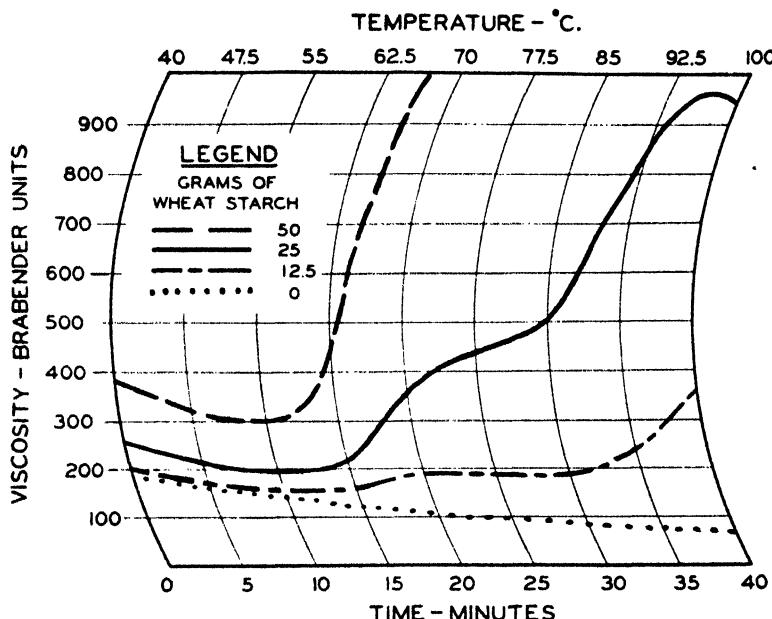


Fig. 3. Effect of various levels of wheat starch on the nature of the curve when gelatinized in 350 g. of 1.25% sodium alginate dispersion.

proper concentration of starch and dispersion medium may prove the existence of stages during gelatinization, it is necessary to examine families of curves to conclude that stages do not exist. Comparison with curves obtained by light transmission studies (2) discloses close agreement in temperatures where first effects of heating are evident.

Differences in Swelling of Various Starches. Fig. 4 shows the curves produced by several types of starches gelatinized in 350 g. of 1.25% sodium alginate dispersion. The quantity of starch used was selected to give best indications of existence of stages for those found to swell by stages. Various levels of tapioca and waxy maize up to 20 g. with concentrations of sodium alginate from 0.75% to 1.25%

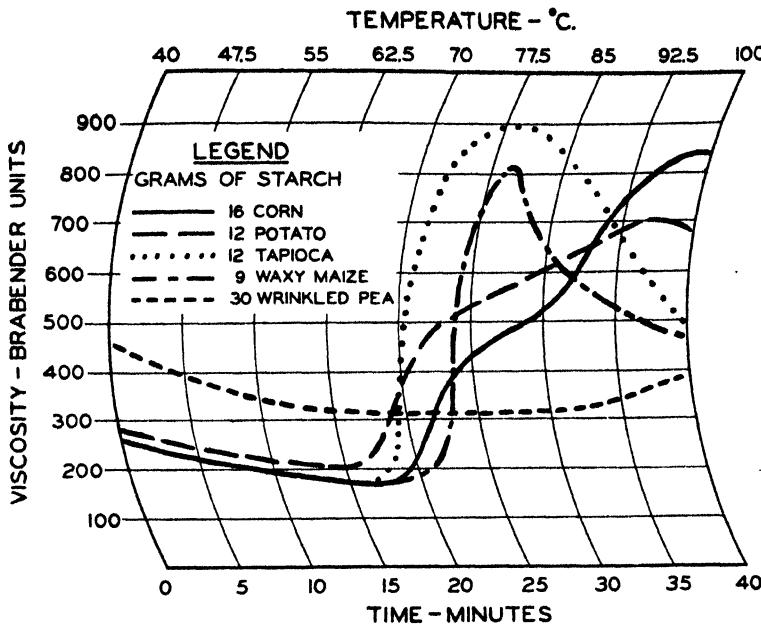


Fig. 4. The nature of curves produced by various types of starch gelatinized in 350 g. of 1.25% sodium alginate dispersion.

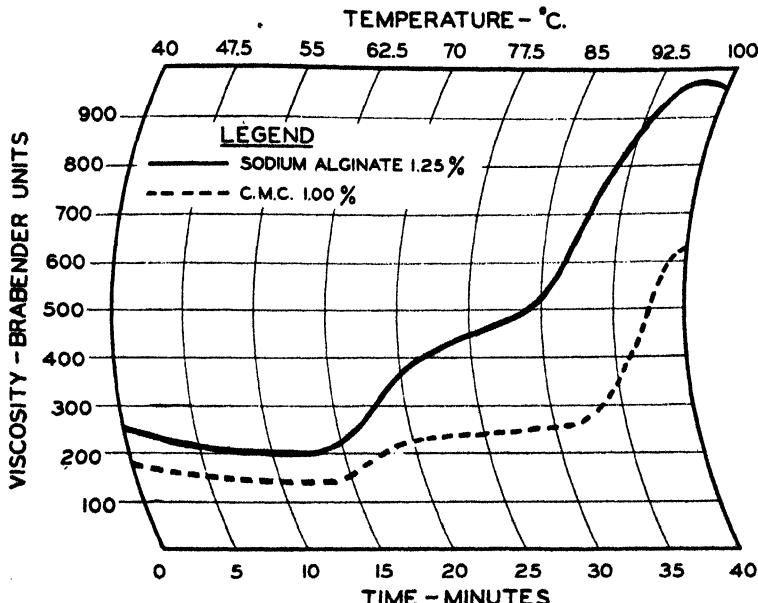


Fig. 5. Comparison of contours of curves produced by gelatinization of 25 g. of wheat starch in solutions of high-viscosity carboxymethylcellulose and sodium alginate.

failed to reveal the existence of stages. For those starches exhibiting only one stage of gelatinization, curves were selected in which the peak viscosity was about 850 Brabender Units.

The curves for corn and potato show the existence of marked differences in the rate of swelling of starch granules in different temperature ranges. The curve for wrinkled pea starch by its comparatively high viscosity indicates that a large amount of water is absorbed initially. The slope of the curve remains fairly constant to a temperature of 55°C. when it goes through zero and slowly becomes greater, indicating that swelling of wrinkled pea starch does not occur at critical temperatures above 40°C. A test with initial temperature at 25°C. also failed to disclose a critical temperature.

Effect of Composition of Dispersion Medium. Fig. 5 shows the similarity between contours of curves produced by gelatinization of 25 g. of wheat starch in 350 g. of 1% solution of high viscosity type carboxymethylcellulose and a 1.25% dispersion of sodium alginate, suggesting that the viscosity changes with temperature shown in the curves of Figs. 2, 3, and 4 are not the result of a specific reaction between the starch and solids of the dispersion medium.

Discussion

It is shown in Fig. 2 that at any temperature the slope is greater as the concentration of the dispersion medium is greater. This relation holds except in the cases where the viscosity exceeds 400 Brabender Units. This exception may be due to either, or a combination, of two effects.

1. High shearing forces causing disintegration of the granules.
2. Increased resistance by the more concentrated dispersion medium to removal of water by the starch, thereby inhibiting maximum swelling of starch granules.

Strong evidence of the action of the first effect is given by the fact that at the highest concentration of dispersion medium a peak viscosity is reached followed by a drop.

Acknowledgment

To Thomas J. Schoch of Corn Products Refining Company, Argo, Illinois, through whose courtesy the sample of wrinkled pea starch was furnished for this study, indebtedness is expressed.

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COMPARATIVE STUDY OF THE EFFECTS OF CYSTEINE HYDROCHLORIDE AND PAPAIN ON UNSALTED AND SALTED DOUGHS¹

ROSA STERN²

ABSTRACT

The effects on doughs of cysteine hydrochloride and papain at two levels of addition were found to be quantitatively different. The amount of reducing matter in the washings from doughs containing papain was practically the same as that in the washings from control doughs, whereas, when cysteine hydrochloride was added to a dough, increasing amounts resulted in increasing quantities of reducing matter in the washings. It is concluded from these findings that the mechanism of cysteine action is different from that of papain action.

Neither cysteine hydrochloride nor papain affected the cystine content of the gluten. Thus, these agents behaved like the thiol compounds and protease present in wheat germ extract.

Washings from unyeasted doughs containing cysteine hydrochloride gave a positive reaction with sodium nitroprusside even after 5 hours dough time; whereas, in the washings from yeasted doughs, the reaction was negative even after 2 hours dough time.

Presence of sodium chloride in dough and washing water led to a decrease of the cystine content of the gluten and of the reducing matter in the washings. Fermentation of salted doughs caused no further decrease in the cystine content of the gluten.

Presence of sodium chloride in dough and wash water checked the gluten breakdown caused by cysteine hydrochloride, and partly so, that due to papain.

The earlier finding of the author that fermentation causes a decrease of the cystine content of the gluten in unsalted doughs was confirmed.

The literature up to 1943 dealing with the effects on dough of oxidizers, reducers, and proteolytic enzymes has been reviewed by Shen and Geddes (5) and by Stern (6). Several papers on this subject have since been published and the more pertinent of them are cited throughout this paper where they have a bearing on points under discussion.

The experiments reported below were undertaken to find further

¹ Manuscript received June 3, 1946.

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evidence as to whether thiol compounds function merely as activators of proteolytic enzymes or whether their action is different from that of these enzymes.

In an earlier investigation Stern (6) studied the separate effects of the protease and the thiol groups naturally present in wheat germ extracts. The present experiments deal with the effects of cysteine hydrochloride and papain added to doughs.

Because of the similarity of the problems the methods applied in the earlier work were also used in the present one. The following reasoning motivated the selection of these methods:

1. If the effects of thiol groups are different from those of proteases of the papain type it might be expected that the amount of soluble protein in the treated doughs and the degree of mechanical breakdown of the gluten caused by one agent would be different from those due to the other. Accordingly, the analytical method was to wash gluten from the doughs and determine the nitrogen content of the washings. Loss of gluten in the washing process was regarded as a measure of mechanical breakdown, and was estimated as the difference between the total nitrogen of the flour and the nitrogen found in the solubles and in the coherent part of the gluten. (Loss of gluten = total nitrogen — soluble nitrogen — nitrogen of coherent gluten.)

2. If, as Sullivan and co-workers (7) suggested, thiol groups react with disulfide groups of gluten, the gluten washed from a dough treated with thiol compounds should contain less cystine than that from a blank dough, because part at least of the disulfide linkages originally present would have undergone chemical change. It should also contain less cystine than gluten from a dough treated with papain which, although containing thiol groups, would supply a comparatively insignificant amount of them. Accordingly, cystine determinations were carried out in the hydrolyzates of the coherent part of glutens washed from treated and untreated doughs. Total cystine (in the coherent and the broken-down fractions) was calculated by the following formula:

$$\text{Total cystine} = \text{cystine found} / (100 - \text{loss of gluten nitrogen}).$$

As discussed in the earlier paper, this formula implies that the cystine content of the coherent part of the gluten is the same as that in the broken-down fraction. It follows that the cystine determined in the coherent fraction should bear the same relation to the cystine present, but not determined, in the disintegrated part as the nitrogen contents of these fractions bear to each other. Significant changes in total cystine (compared with the figures applying to gluten from an unyeasted blank dough) would indicate that the treatment caused either

a change in the cystine content of the gluten or a change in the cystine distribution between coherent and disintegrated fractions.

In addition to the determinations discussed above, the reducing matter in the dough solubles was estimated, and tests with sodium nitroprusside were carried out.

The experiments, with a view to practical baking conditions, covered salted as well as unsalted doughs.

Materials and Methods

Materials. The flour used was a commercial straight run flour milled from New Zealand wheat. It contained 15.7% moisture, 0.40% ash, and 8.1% protein, and gave a satisfactory mature loaf when baked according to the A.A.C.C. formula. Some of the experiments were repeated on other flours, but as the results obtained were not basically different they will not be reported here.

The cysteine hydrochloride was a preparation from Hoffmann LaRoche and Company. The papain was a Kahlbaum product; it contained 264 mg. nitrogen per g. and reduced 2.38 ml. *N*/100 iodine solution per g.

Methods. The gluten washing process was carried out by hand, using 10 separate portions of tap water, or of 2% sodium chloride solution. No silk was used.

The gluten recovered as a coherent lump was torn into small pieces and hydrolyzed in approximately 6 *N* sulfuric acid solution for 18 hours.

Total nitrogen was determined in the flour and in the coherent part of the gluten (by analyzing an aliquot of the unfiltered gluten hydrolysate). Soluble nitrogen was determined in an aliquot of the filtered combined washings, the volume of which was assumed to be 514 ml. (500 ml. water used for washing, plus 15 ml. dough liquid, plus 3.9 ml. flour moisture, minus 4.7 ml. hydration water of wet gluten). The mechanical loss due to gluten breakdown was found by deducting the nitrogen of coherent gluten plus soluble nitrogen from the total nitrogen and was expressed in per cent of total gluten nitrogen.

Cystine was determined in aliquots of the filtered gluten hydrolysates. The method of Mirsky and Anson (4) was used, and extraneous reducers were determined according to Lugg (3). In applying the technique of Mirsky and Anson, however, urea was not employed. The values obtained in absence of urea were, on the average, 15% lower than when urea was present, but the trend within each series of experiments was the same. Total cystine was computed by the formula: total cystine = cystine found/(100 - % gluten nitrogen lost) (Stern, 6).

Reducing matter in the washings was determined as follows: As soon as the washing process was finished, the combined washings were thoroughly mixed, and roughly 150 ml. of the suspension were centrifuged. Portions of 100 ml. of the centrifugate were deproteinized with sulfuric acid and sodium tungstate (*Cereal Laboratory Methods*, 1941, p. 100), and again centrifuged. To 100 ml. of this second centrifugate (corresponding to 92.6 ml. of the first one) 5 ml. or, if necessary, 10 ml. of *N* 500 iodine solution were added, and the excess was backtitrated with *N* 500 sodium thiosulfate solution.

The nitroprusside reaction was carried out in the washings obtained from the first 50 ml. portion of water or salt solution used, as the concentration of thiol groups in the combined washings is too low to give a positive reaction. The suspension resulting from washing with this first portion of washing liquid was centrifuged, a few ml. of the centrifugate were saturated with sodium chloride, acidified with acetic acid, three drops of a 5% solution of sodium nitroprusside were added, and the contents of the test tube made slightly alkaline with ammonia. A color change to a purple or purplish hue indicated the presence of thiol groups.

Results

Experiments with Unsalted Doughs Washed with Tap Water. The doughs for the first series of experiments were made from 25 g. of flour mixed with 15 ml. of tap water, and tap water was also used in washing out the gluten. When the doughs were to be fermented, 0.5 g. of yeast was suspended in the dough liquid. Cysteine treatment involved addition of 2.55 mg. cysteine hydrochloride (0.01% on flour basis), and papain treatment involved addition of 7.5 mg. papain (0.03% on flour basis).³ Dough times of 1, 3, and 5 hours were used, and all doughs were maintained at 27.8°C. Table I shows the results obtained in these experiments.

Unfermented Doughs. The data indicate that only papain caused an appreciable increase in soluble nitrogen with dough time. Gluten losses increased with dough time at similar rates in both cysteine and papain doughs. The cystine content of the gluten was independent of dough time. An analysis of variance showed that neither cysteine hydrochloride nor papain significantly affected the cystine content of gluten. The reducing matter in the deproteinized washings from the cysteine doughs was very much in excess of that in the washings from either blank or papain doughs. The reducing matter content of

³ The additions of cysteine hydrochloride and papain were chosen so as to cause moderate and approximately equal damage to the gluten on washing, at the lower level of addition. In the baking test, the deterioration due to corresponding additions was much more severe in the papain dough than in the cysteine dough. The concentrations used lie within the limits of additions applied by other workers.

the last two was practically the same. The nitroprusside reaction of the washings from the cysteine doughs was positive after 5 hours dough time.

Fermented Doughs. Soluble nitrogen was higher than in the unfermented series and, in all doughs, increased with dough time. There was, however, no significant difference between the soluble nitrogen in cysteine doughs and in papain doughs at the level of addition employed. The gluten losses were also much larger than in the unfermented series and failed to reveal a difference between treatment

TABLE I

EFFECTS OF CYSTEINE HYDROCHLORIDE AND PAPAIN AT VARYING DOUGH TIMES ON UNYEASTED AND YEASTED DOUGHS, UNSALTED AND WASHED WITH TAP WATER

(Dough times of 1, 3, and 5 hours at 27.8°C.
Total nitrogen in 25 g. flour = 357 mg.)

Determination	Dough-time hrs.	Without yeast			With yeast		
		control	cysteine	papain	control	cysteine	papain
Nitrogen in coherent gluten, mg.	1	300	289	292	279	261	273
	3	289	273	272	220	152	148
	5	293	269	249	177	103	87
Soluble nitrogen, mg.	1	48	52	50	59	72	65
	3	51	56	56	78	86	84
	5	49	55	60	88	97	96
Lost nitrogen in % of total gluten-nitrogen	1	2.9	5.2	4.9	6.4	8.4	6.5
	3	5.6	9.3	9.6	21.1	43.9	45.8
	5	4.9	10.9	18.5	34.2	60.4	66.7
Total cystine in gluten, mg.	1	44.0	45.0	44.6	43.6	42.7	45.1
	3	44.6	42.1	43.9	40.8	38.7	40.6
	5	43.5	45.1	45.6	40.9	41.9	43.2
Reducing matter, as ml. N/500 iodine solution	1	1.7	11.6	1.5	3.4	13.4	2.3
	3	1.7	11.2	1.4	4.2	16.5	4.8
	5	2.8	12.8	2.6	3.7	14.3	3.4

with cysteine and papain. The cystine content of the glutens was not affected by either of the additions. Dough fermentation, however, caused a significant decrease of cystine in the glutens from all the doughs. Both these observations are in keeping with the results obtained in the earlier work on the effects of wheat germ. Reducing matter was generally higher than in the unfermented doughs, and here again the figures for the cysteine doughs were very much higher than those for either the papain or control dough. The nitroprusside reaction in the washings from the cysteine doughs was weak after 1 hour and negative after 2 hours dough time.

In summary, the results contained in Table I indicate that, at the applied level of additions, the only significant difference between cysteine and papain treatments was the content of reducing matter in the respective doughs. The experiments revealed that, independent of any additions, dough fermentation lowered the cystine content of gluten.

A further series of experiments involved additions of cysteine hydrochloride and papain at a level 100% higher than that applied in the first series. Table II gives a comparative summary of the values obtained at both levels of addition.

TABLE II

EFFECTS OF CYSTEINE HYDROCHLORIDE AND PAPAIN, AT TWO LEVELS OF ADDITION, ON UNYEASTED AND YEASTED DOUGHS,
UNSALTED AND WASHED WITH TAP WATER

(Dough time 3 hours at 27.8°C. Total nitrogen in 25 g. flour = 357 mg.)

Determination	Without yeast				With yeast			
	cysteine		papain		cysteine		papain	
	2.55 mg.	5.1 mg.	7.5 mg.	15.0 mg.	2.55 mg.	5.1 mg.	7.5 mg.	15.0 mg.
Nitrogen in coherent gluten, mg.	273	161	272	28	152	143	148	4
Soluble nitrogen, mg.	56	66	56	90	86	93	84	133
Lost nitrogen in % of total gluten-nitrogen	9.3	44.7	9.6	89.5	43.9	45.8	45.8	98.2
Total cystine in gluten, mg.	42.1	46.5	43.9	44.4	38.7	35.2	40.6	38.9
Reducing matter, as ml. <i>N</i> /500 iodine solution	11.2	25.1	1.4	1.7	16.5	30.9	4.8	4.5

Unfermented Doughs. Soluble nitrogen as well as gluten losses increased with increasing additions. At the higher level, these increases were much larger in the papain than in the cysteine doughs. Total cystine was again unaffected by either agent.

Reducing matter in the washings from the cysteine doughs increased roughly in proportion to the increased addition, whereas it remained practically constant in the washings from the papain doughs and controls.

Fermented Doughs. Soluble nitrogen increased from the lower to the higher level of addition, much more so in the papain doughs than in the cysteine doughs. Gluten losses on washing were but little in-

fluenced by the increased addition of cysteine hydrochloride, whereas the increased addition of papain caused almost complete breakdown of the gluten. The gluten breakdown caused by cysteine hydrochloride does not seem to proceed beyond a certain limit. This observation is in keeping with the findings of Ford and Maiden (2), Swanson and Andrews (8), and of Balls and Hale (1).

Total cystine did not respond to increased additions of either cysteine hydrochloride or papain. Fermentation again lowered the cystine content of the gluten. Reducing matter in the cysteine doughs, as in the unfermented series, increased roughly in proportion to the addition made.

Experiments with Salted Doughs Washed with Salt Solution. The doughs for this series of experiments were made from 25 g. of flour mixed with 15 ml. of tap water containing 0.5 g. of salt. When the doughs were to be fermented, 0.5 g. of yeast was suspended in the dough. A 2% solution of commercial salt was used for washing out the gluten. The additions were 2.55 mg. of cysteine hydrochloride or 7.5 mg. of papain. The doughs were kept at 27.8°C. for 3 hours.

Table III shows the results obtained in this series.

TABLE III

EFFECTS OF CYSTEINE HYDROCHLORIDE AND PAPAIN ON UNYEASTED AND YEASTED DOUGHS, SALTED AND WASHED WITH A 2% SODIUM CHLORIDE SOLUTION

(Dough time 3 hours at 27.8°C. Total nitrogen in 25 g. flour = 357 mg.)

Determination	Without yeast			With yeast		
	control	cysteine	papain	control	cysteine	papain
Nitrogen in coherent gluten, mg.	281	282	275	283	285	190
Soluble nitrogen, mg.	64	69	70	58	59	76
Lost nitrogen in % of total gluten	4.1	2.1	4.2	5.3	4.4	32.4
Total cystine, mg.	41.9	40.8	38.5	40.8	42.7	41.1
Reducing matter, as ml. N/500 iodine solution	0	7.2	0	0	10.9	0

The values for soluble nitrogen and loss of gluten are different from the corresponding values obtained in absence of salt, being higher in the unfermented and lower in the fermented series. A significant increase in soluble nitrogen and loss of gluten occurred only in the fermented dough containing papain. The cystine content of the glutens was unaffected by either cysteine hydrochloride or papain.

Reducing matter disappeared from the washings of all doughs except those containing cysteine hydrochloride.

The nitroprusside test was positive in the unyeasted and negative in the yeasted cysteine doughs.

The use of sodium chloride resulted in lowering the cystine content of the glutens from all doughs, and in eliminating the difference between unyeasted and yeasted doughs which was observed in absence of salt.

Discussion

As previously stated, the objective of these experiments was to secure data which would aid in elucidating the mechanism of the action of cysteine and papain in doughs.

At the lower levels of addition, the effects of cysteine hydrochloride and papain, expressed in terms of soluble nitrogen and gluten breakdown, were approximately equal, but at the higher levels papain produced much more soluble nitrogen and caused a far severer breakdown of the gluten. These observations are in agreement with the findings of Swanson and Andrews (8) who concluded that the mechanism of papain action is different from that of cysteine action.

The figures for soluble reducing matter also indicate that cysteine hydrochloride affects the dough in a different way from papain. Doughs containing either of these agents in amounts causing approximately equal damage yielded washings which differed widely in their contents of reducing matter. The quantity of reducing matter washed from cysteine doughs was a multiple of that present in the washings from papain doughs. The further observation that the reducing matter washed from cysteine doughs was roughly proportional to the amount of cysteine added suggests that the cysteine hydrochloride (or the bulk of it) was released by the washing process, or that some other water-soluble reducing substance was formed. In either case, the linkage between gluten proteins and cysteine must have been a loose one. That disulfide linkages of the gluten were not involved in this linkage is borne out by the fact that the cystine content of the gluten remained unchanged. The gradual improvement which doughs containing thiol compounds undergo on prolonged fermentation would indicate that the bond between thiol compounds and gluten proteins is such that it leaves the thiol group free to be oxidized or otherwise to react. The nitroprusside test showed that thiol groups gradually disappear in a fermenting dough, but this does not result in a decrease of reducing matter. It would appear that the reducing matter washed from unyeasted cysteine doughs was unchanged cysteine hydrochloride, whereas the reducing matter washed from yeasted doughs was a re-

ducing compound deriving from cysteine, perhaps the sulfenic or sulfinic acid.

These conclusions were drawn from values which show a similar trend in both unsalted and salted series. The differences between those two series, involving not only soluble nitrogen and gluten losses, but also reducing matter and cystine content, are probably all attributable to changes in protein solubility in the presence of sodium chloride.

Acknowledgments

The author wishes to record her grateful appreciation of Peggy Aiken's able and untiring help in the analytical part of this investigation. Her thanks are also due to Sheila Boyce for advice and help in the application of statistical methods.

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SUGGESTIONS TO AUTHORS

General. From January 1, 1948, an abstract will be printed at the beginning of each paper instead of a summary at the end, references will be numbered to provide the option of citing by number only, and date of receipt, author's connections, etc., will be shown in footnotes. Except on these points, authors will find the last volume of *Cereal Chemistry* a useful guide to acceptable arrangements and styling of papers. "On Writing Scientific Papers for Cereal Chemistry" (*Trans. Am. Assoc. Cereal Chem.* 6: 1-22. 1948) amplifies the following notes.

Authors should submit two copies of the manuscript, typed double spaced with wide margins on 8½ by 11 inch white paper, and all original drawings or photographs for figures. If possible, one set of photographs of figures should also be submitted. Originals can then be held to prevent damage, and the photographs can be sent to reviewers.

Titles and Footnotes. Titles should be specific, but should be kept short by deleting unnecessary words. The title footnote shows "Manuscript received . . ." and the name and address of the author's institution. Author footnotes, showing position and connections, are desirable although not obligatory.

Abstract. A concise abstract of about 200 words follows title and authors. It should state the principal results and conclusions, and should contain, largely by inference, adequate information on the scope and design of the investigation.

Literature. In general, only recent papers need be listed, and these can often be cited more advantageously throughout the text than in the introduction. Long introductory reviews should be avoided, especially when a recent review in another paper or in a monograph can be cited instead.

References are arranged and numbered in alphabetical order of authors' names and show author, title, journal, volume, first and last pages, and year. The list is given at the end of the paper. Reference numbers must invariably be cited in the text, but authors' names and year may be cited also. Abbreviations for the names of journals follow the list given in *Chemical Abstracts* 40: I-CCIX. 1946.

Organization. The standard organization involves main sections for abstract, introduction, materials, methods, results, discussion, acknowledgments, and literature cited. Alternately, a group of related studies, each made with different materials or methods, may require a separate section for each study, with subsections for materials and methods, and for results, under each section. Center headings are used for main sections and italicized run-in headings for subsections, and headings should be restricted to these two types only.

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Tables should be typed on separate pages at the end of the manuscript, and their position should be indicated to the printer by typing "(TABLE I)" in the appropriate place between lines of the text. (Figures are treated in the same way.)

Figures. If possible, all line drawings should be made by a competent draftsman. Traditional layouts should be followed: the horizontal axis should be used for

the independent variable; curves should be drawn heaviest, axes or frame intermediate, and the grid lines lightest; and experimental points should be shown. Labels are preferable to legends. Authors should avoid identification in cut-lines to be printed below the figure, especially if symbols are used that cannot readily be set in type.

All drawings should be made about two to three times eventual reduced size with India ink on white paper, tracing linen, or *blue*-lined graph paper; with any other color, the unsightly mass of small grid lines is reproduced in the cut. Lettering should be done with a guide using India ink; and letters should be $\frac{1}{8}$ to $\frac{1}{4}$ th inch high after reduction.

For difficult photographs, a professional should be hired or aid obtained from a good amateur. The subject should be lighted to show details. A bright print with considerable contrast reproduces best, and all prints should be made on glossy paper.

All original figures should be submitted with one set of photographic reproductions for reviewers, and each item should be identified by lightly writing number, author, and title on the back. Cut-lines (legends) should be typed on a separate sheet at the end of the manuscript. "Preparation of Illustrations and Tables" (*Trans. Am. Assoc. Cereal Chem.* 3: 69-104. 1945) amplifies these notes.

Text. Clarity and conciseness are the prime essentials of a good scientific style. Proper grouping of related information and thoughts within paragraphs, selection of logical sequences for paragraphs and for sentences within paragraphs, and a skillful use of headings and topic sentences are the greatest aids to clarity. Clear phrasing is simplified by writing short sentences, using direct statements and active verbs, and preferring the concrete to the abstract, the specific to the general, and the definite to the vague. Trite circumlocutions and useless modifiers are the main causes of verbosity; they should be removed by repeated editing of drafts.

Editorial Style. A.A.C.C. publications are edited in accordance with *A Manual of Style*, University of Chicago Press, and *Webster's Dictionary*. A few points which authors often treat wrongly are listed below:

Use names, not formulas, for text references to chemical compounds. Use plural verbs with quantities (6.9 g. were). Figures are used before unit abbreviations (3 ml.), and % rather than "per cent" is used following figures. All units are abbreviated and followed by periods, except units of time, which are spelled out. Repeat the degree sign (5°-10° C.). Place 0 before the decimal point for correlation coefficients ($r = 0.95$). Use * to mark statistics that exceed the 5% level and ** for those that exceed the 1% level; footnotes explaining this convention are no longer required. Type fractions on one line if possible, e.g., $A/(B + C)$. Use lower case for farinograph, mixogram, etc., unless used with a proper name, i.e., Brabender Farinograph. When in doubt about a point that occurs frequently, consult the Style Manual or the Dictionary.

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A COMPARATIVE STUDY OF SOME PROTEIN FRACTIONS OF WHEAT FLOUR¹

W. DERBY LAWS² and W. G. FRANCE³

ABSTRACT

An electrophoresis investigation of some protein fractions of flour from various wheats was carried out with the idea that it might reveal the presence of different protein components in the gluten complex. It proved impractical to study the proteins in either alcoholic buffers or in aqueous alkaline buffers with a pH of 10.2. Therefore, attention was turned to the electrophoretic study of gluten proteins dissolved in acetic acid and dialyzed against citric acid-disodium phosphate buffer, pH 2.15. However, this procedure failed to reveal any significant difference in the flour proteins. Electrophoresis of the water extract of Chiefkan, Early Blackhull, and Comanche flours gave similar electrophoretic patterns for all flours.

Baking tests with flour fortified with wet and dry gluten washed from the various flours appear to confirm the results obtained by electrophoresis technique, namely, that the glutens from the flours studied are very similar in properties. When flour blends were fortified with wet, freshly washed gluten, the Chiefkan gluten gave the greatest increase in loaf volume and the Comanche the least for a given amount of protein added.

This work was one phase of an investigation of the factors and constituents in wheat which are responsible for quality in flour. The general plan of the investigation was to compare the characteristics of poor quality flour *vs.* good quality flour. Chiefkan and Red Chief wheats were selected as varieties which generally give poor quality flour, Pawnee and Comanche wheats as the varieties which generally yield good quality flour, and Early Blackhull and Wichita wheats as the varieties which are probably intermediate between the other two groups. In order to make the samples comparable they were collected in triplicates, one member of each type from a given location, and each set of three collected from a different location so that samples were

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obtained from three widely separated areas. The samples collected, the location from which obtained, and the baking quality of each are presented in Table I.

Cereal chemists generally agree that the properties of wheat gluten determine flour quality. Consequently, many attempts have been made to find measurable differences in the protein fractions which could be shown to be responsible for the difference in the quality of the flour. An electrophoretic investigation of some protein fractions of the flour was undertaken with the idea that it might reveal the presence of different protein components in the flour from the three groups of wheat being studied.

TABLE I
WHEAT SAMPLES STUDIED

Variety	Location	Designation	Baking quality
Chiefkan	Protection, Kansas	Cp	Very poor
Early Blackhull	Protection, Kansas	Bp	Fair
Comanche	Protection, Kansas	Hp	Fair
Chiefkan	Jetmore, Kansas	Cj	Very poor
Early Blackhull	Jetmore, Kansas	Bj	Poor
Comanche	Jetmore, Kansas	Hj	Poor
Red Chief	Newton, Kansas	Rn	Very poor
Wichita	Newton, Kansas	Wn	Fair
Pawnee	Newton, Kansas	Pn	Poor
Early Triumph	Caldwell, Kansas	Ec	Good

The electrophoresis apparatus suitable for the quantitative study of proteins by the moving boundary method has been much improved and widely used until it has become one of the most powerful tools available to biological chemists for the study of protein systems. The moving boundary electrophoresis method is applicable to a wide variety of high molecular weight substances, both in their native and denatured form, and yields information as to the number of electrically separable components present in the mixture and the electrical homogeneity, concentration, and mobility of each component. Since this method is adapted to the study of a wide variety of proteins and protein mixtures in dilute aqueous buffers, it seemed to offer the best means of detecting differences in the proteins of good quality and poor quality flours despite the lack of suitable neutral solvents for gluten proteins.

The present investigation was not an attempt to determine the definite composition of the gluten proteins from the various flours but rather to make a comparative study of the glutens from each flour.

Experimental

No attempt was made to separate the gluten into fractions for this study. Instead, either the whole gluten mass or protein extracted directly from the flour was used. A Tiselius electrophoresis apparatus manufactured by the Klett Manufacturing Company was used for the electrophoretic determinations.

Electrophoretic determinations were made on the protein fractions of flour from each series of wheat samples presented in Table I.

The preliminary determinations were made using alcoholic buffers which contained 50% of ethyl alcohol by volume. These buffers were

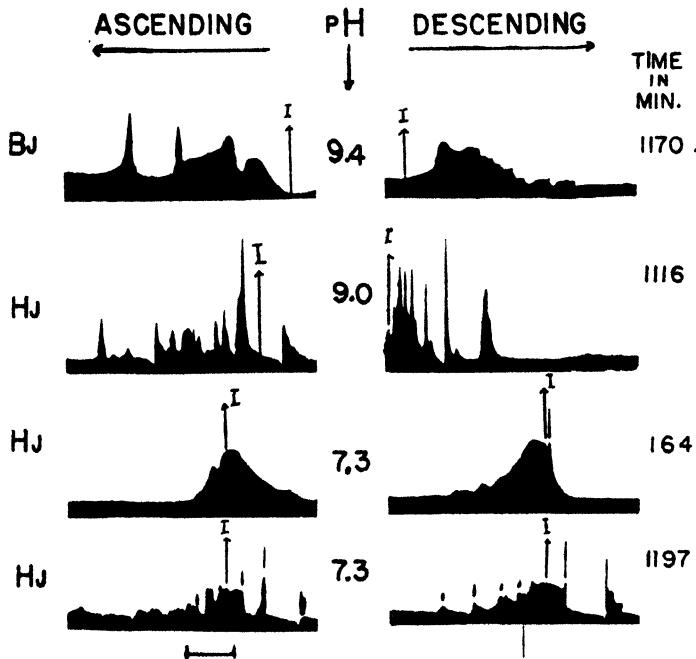
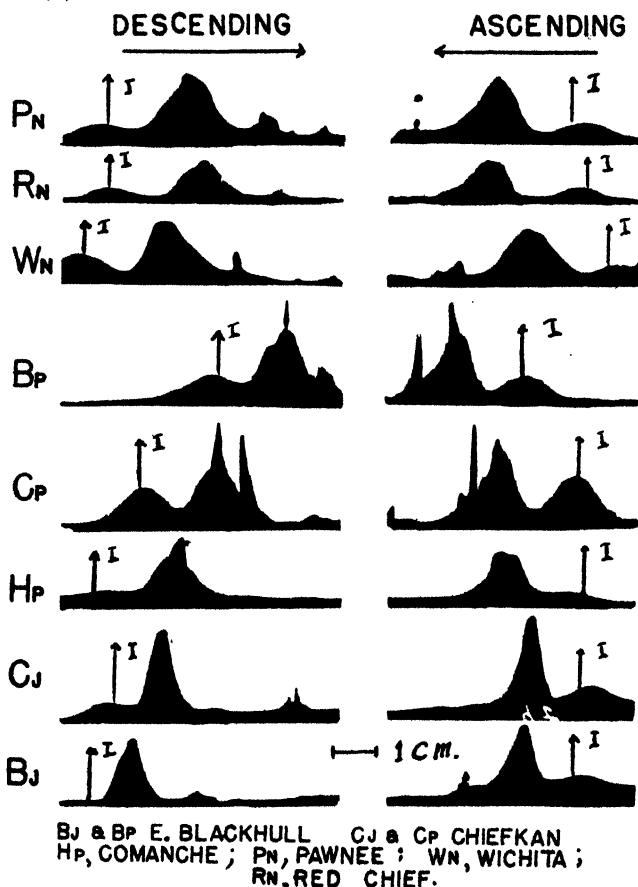


Fig. 1. Electrophoresis patterns of alcohol-soluble flour proteins in 50% alcoholic buffers. (Bj field strength 8.0 volts/cm., Hj (pH 9.0) 9.2 volts/cm.; Hj (pH 7.3) 8.3 volts/cm. "I" indicates initial boundary. Line equals 1 cm. Descending boundary at negative (-) pole.)

prepared by diluting aqueous buffers prepared after the manner of Clark and Lubs with sufficient 95% alcohol to give a solution of desired alcoholic concentration. An aqueous buffer of pH 8.0, when thus diluted, resulted in a buffer of pH 9.0-9.4, and one of pH 6.0 gave an alcoholic buffer of pH 7.3.

The method of making all the electrophoretic determinations was essentially as described in the literature (1) (6). Runs were made at a temperature of 2.5°C. As a general rule it required from 10 to 20 hours at a field strength of about 8.5 volts per cm. to complete a de-

termination in alcoholic buffers. Although patterns were obtained (Fig. 1) no mobilities were calculated because, in addition to the difficulty of determining the pH and ionic strength of the buffer in alcoholic medium, the long periods of electrophoresis and the enforced study at a temperature above that of maximum density of alcohol-water mixtures result in a marked increase in boundary disturbances due to thermal convection (8).⁴



B_J & B_P E. BLACKHULL C_J & C_P CHIEFKAN
H_P, COMANCHE; P_N, PAWNEE; W_N, WICHITA;
R_N, RED CHIEF.

Fig. 2. Electrophoresis patterns of gluten proteins from Hard Red Winter wheats. (Boric acid-sodium hydroxide buffer pH 10.2. X = field strength, T = time in minutes. For P_N, X = 3.87, T = 269; R_N, X = 3.99, T = 258; W_N, X = 3.87, T = 262; B_P, X = 4.06, T = 205; C_P, X = 4.17, T = 205; H_P, X = 4.09, T = 214; C_J, X = 3.61, T = 198; B_J, X = 3.51, T = 203. "I" indicates the initial boundary. Line equals 1 cm. Descending boundary at negative (-) pole.)

Since the above procedure was not satisfactory, attention was turned to electrophoretic studies in aqueous buffers. Cook and Rose (3) (4) have shown that gluten can be almost completely dissolved

⁴ The above paper was published after our work with alcoholic buffers had been discontinued in January, 1947.

in 10% sodium salicylate without undergoing hydrolytic changes on long standing. Accordingly, a 10% solution of sodium salicylate was selected as a solvent. A sodium hydroxide-boric acid buffer with a pH 10.2 (original ionic strength, 0.094) prepared after the manner of Clark and Lubs was used. The flour samples from the three locations were studied and the patterns obtained are presented in Fig. 2.

Although it is well known that gluten proteins undergo irreversible denaturation in alkaline solution, it seemed probable that any marked

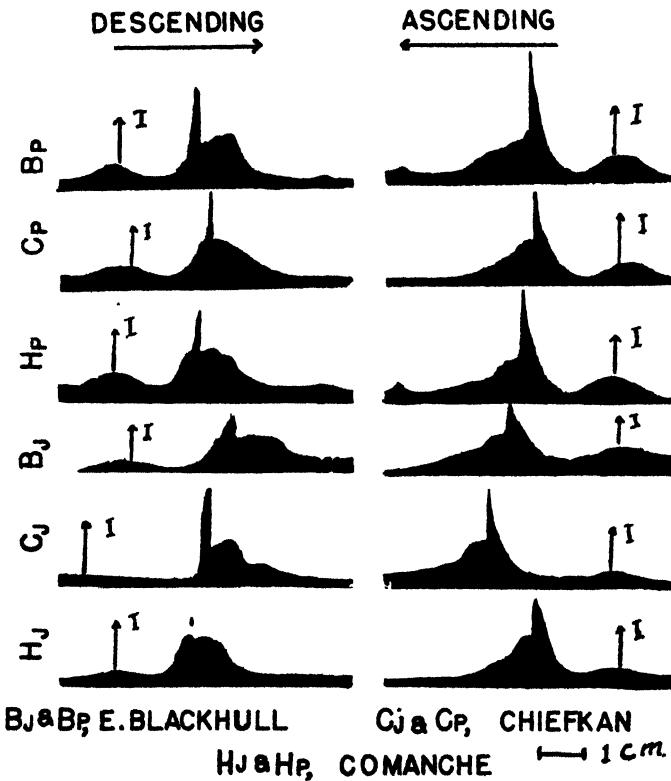


Fig. 3. Electrophoresis patterns of gluten proteins from Hard Red Winter wheats. (Boric acid-sodium hydroxide buffer pH 10.2. Ionic strength 0.047. Field strength \times time equals 67,500 volt-sec./cm. for Cp, Hp, and Bp; 91,560 for Bj; 96,620 for Cj; and 59,320 for Hj. "I" indicates initial boundary. Line equals 1 cm. Descending boundary at negative (-) pole.)

difference in the protein composition of the gluten from the various flours would appear in the electrophoresis patterns as additional components even though the proteins were denatured. Since this investigation did not try to characterize the various components of the gluten proteins but merely attempted to compare the number of electrically separable components present in the glutens of good quality and poor quality flour, it would seem to matter little whether the protein com-

ponents were denatured or not so long as they remained electrically separable. (For present purposes it was necessary to assume that they do.)

The ionic strength of the buffer was about twice as high as considered necessary for protein solutions of the concentration used, which varied from 0.3% to 0.4%. Therefore, a study was made of gluten from some of the same samples, using the same buffer with the ionic strength reduced to 50% of the original value. The diagrams obtained are presented in Fig. 3.

In both cases the samples were prepared by stirring 7.5 g. of wet, freshly washed gluten in 250 ml. of 10% sodium salicylate, in a Waring Blender, for 5 minutes, centrifuging for 10 minutes at a centrifugal acceleration 400 times gravity to destroy the foam and remove traces of starch, and dialyzing 100 ml. for 48 hours against 2 liters of the respective buffers. Dialysis was carried out in the cold room at 2°C. The buffer was not changed during dialysis but the protein solution was in equilibrium with the buffer in every case as shown by the measurement of the specific conductance of each at the beginning of the run. Just before placing in the electrophoresis cell the sample was again centrifuged for 10 minutes, in the cold room, to remove the material which salted out.

It should be noted that in all these electrophoretic determinations only about 40% of the gluten added remained in suspension after dialysis. The data in Table II show the amount for the individual samples.

TABLE II

PORTION OF ADDED GLUTEN REMAINING IN SUSPENSION AFTER DIALYSIS AGAINST
A SODIUM HYDROXIDE-BORIC ACID BUFFER, pH 10.2

Variety	From Jetmore	From Protection	Variety	From Newton
Chiefkan	45.7	37.2	Red Chief	44.1
Early Blackhull	42.5	40.5	Wichita	45.6
Comanche	36.7	35.7	Pawnee	39.0

Since it was not possible to control the composition of the sample, making it necessary to assume that the same protein fraction remained in suspension for each sample and on each duplicate run, a study of various buffer systems was made and it was found that a citric acid-disodium phosphate buffer was more satisfactory because a greater portion of the added gluten remained in suspension after dialysis. The data in Table III show the portion of gluten in the original acetic acid suspension remaining in solution after 48 hours dialysis.

The procedure used in preparing the sample was as follows: 7.5 g. of freshly washed, wet gluten were suspended in 250 ml. of 0.07 *N* acetic acid by stirring for 5 minutes in a Waring Blender. The suspension was then centrifuged for 10 minutes at an acceleration 400 times gravity to remove traces of starch and destroy the foam formed

TABLE III

PORTION OF ADDED GLUTEN REMAINING IN SUSPENSION AFTER DIALYSIS AGAINST CITRIC ACID-DISODIUM PHOSPHATE BUFFER, pH 2.15

Sample	% Protein for gluten	% Protein for flour
Chiefkan (Cj)	88.6	91.2
Early Blackhull (Bj)	93.7	—
Comanche (Hj)	96.1	92.0
Early Triumph (Ec)	93.4	98.3

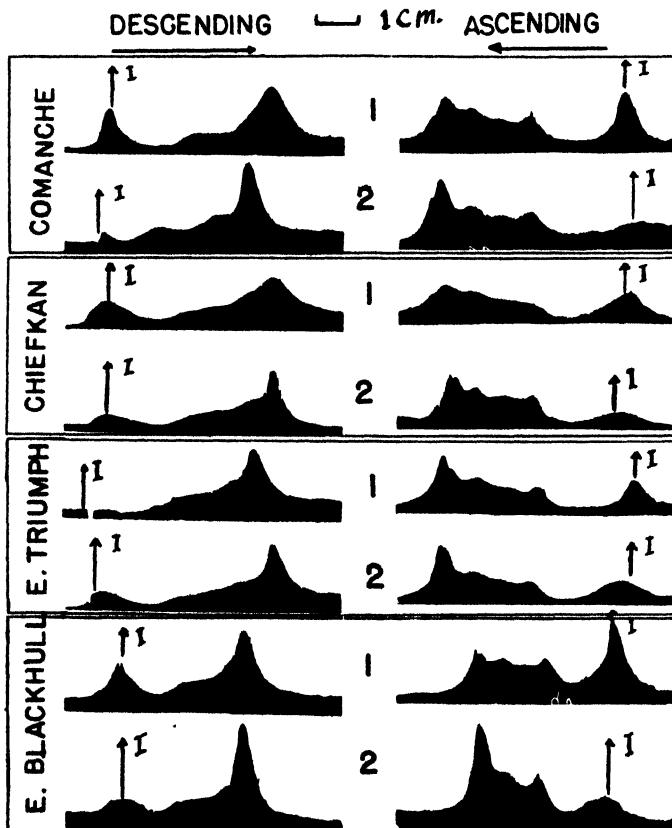


Fig. 4. Electrophoresis patterns of Hard Red Winter wheat protein. Picture No. 1 for protein extracted directly from flour and No. 2 for dissolved gluten. (Citric acid-disodium phosphate buffer, pH 2.15 and ionic strength 0.03. Field strength \times time = 85,320 volt-sec./cm. for Comanche, Chiefkan, and Early Triumph and 66,400 volt-sec./cm. for Early Blackhull. "I" indicates initial boundary. Line equals 1 cm. Descending boundary at positive (+) pole.)

during stirring. Of the suspension thus obtained 100 ml. were then heated to 92°C. and held there for 2 minutes to destroy the proteolytic enzymes present. It has been shown that this treatment does not denature the protein (7). After heating, the sample was diluted sufficiently with buffer to give a solution of 0.26% protein. It (100 ml.) was then dialyzed against 2 liters of a citric acid-disodium phosphate

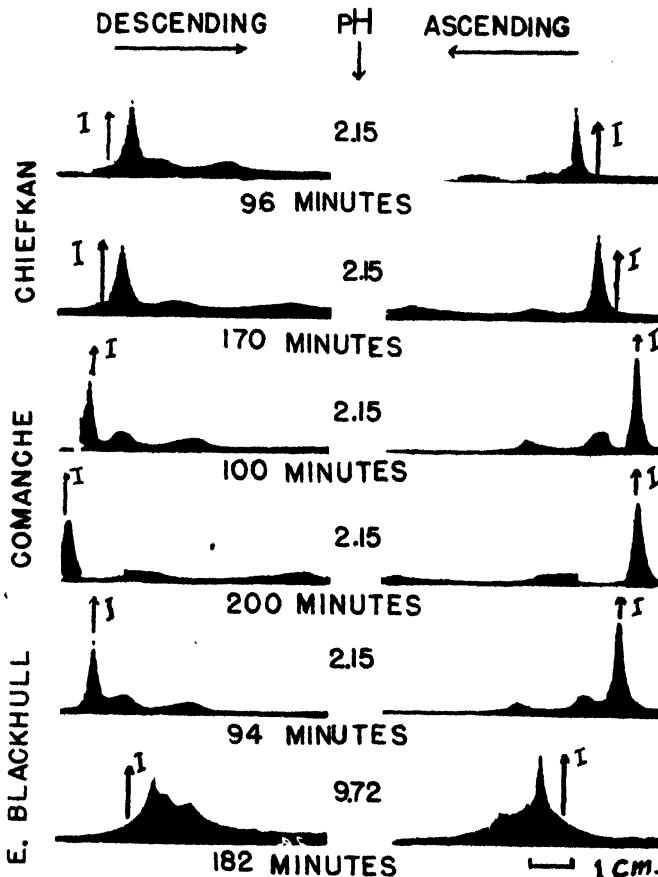


Fig. 5. Electrophoresis patterns of water-extractable proteins from Hard Red Winter wheat flour. (Field strength for Chiefkan 4.21, Comanche 4.24, E. Blackhull (pH 2.15) 4.20, and (pH 9.72) 4.26 volts/cm. Ionic strength 0.03. "I" indicates initial boundary. Line equals 1 cm. Descending boundary at positive (+) pole. For E. Blackhull, pH 9.72, descending boundary at negative (-) pole.

buffer of pH 2.15 and an ionic strength of 0.03 in the cold room (2°C.) until equilibrium between buffer and sol was reached as shown by conductivity measurements (72 hours), and centrifuged again just before placing in the electrophoresis cell. The patterns obtained are presented in Fig. 4. The top pattern, No. 1, of each pair in Fig. 4 was made of protein extracted directly from the flour. In this case

20 g. of flour were stirred in 250 ml. of 0.07 *N* acetic acid; otherwise the procedure used in preparing the samples was exactly as described above. The time for all runs was such that the product of the field strength and the time was a constant, as suggested by Schwert *et al.* (9). The system was tested for electrical leaks at the beginning and end of each run. At the conclusion of a run the current was reversed and the movement of the components observed 1½ hours. Any leak in the cell causing backward movement of the boundary would be greatly amplified by this procedure and thus readily detected.

The work of Finney (5) and also observations made in this laboratory indicate that the water-soluble protein may be more important to flour quality than has formerly been supposed. Therefore, an electrophoresis study was made of *distilled water extracts of the flour* using the citric acid-disodium phosphate buffer described above. The patterns obtained are presented in Fig. 5. All flour samples gave patterns that are alike and that have the same number of components. The Early Blackhull sample was also run at pH 9.72 and gave an electrophoresis pattern entirely different from that obtained for the same sample at pH 2.15.

Discussion

The patterns shown in Fig. 3 are essentially the same for all six flour samples studied and there was little similarity between patterns of proteins from the same flour sample run at different ionic strength (see Fig. 2 and Fig. 3). The higher ionic strength appears to be best suited to a study of this type since there is evidence of better component separation in the patterns in Fig. 2 than in Fig. 3.

There was no essential difference in the patterns obtained from the various flour samples when an acidic buffer, pH 2.15, was used. The descending and ascending boundaries (Fig. 4) were not symmetrical, indicating component interaction. The patterns for protein extracted directly from the flour and for gluten were very nearly identical except for the slow-moving component which was much more prominent on the patterns made from the protein extracted directly from the flour. This was probably due to the presence of part of the water-soluble fraction in the suspension, as it was shown that the main component from the water-soluble protein has a very low mobility also. There would be little water-soluble protein present in the gluten suspension because of the method of sample preparation.

The slow-moving "component" moved so very little that it could easily be taken for the well-known delta and epsilon anomalous boundaries except for the fact that the area under the remainder of the peaks (for the patterns of protein extracted directly from flour, No. 1, Fig. 4)

only accounts for about 80% of the protein present in the solution, and the area of the first peak must be taken into consideration in order to account for all the protein present. This is shown clearly in Table IV. These data indicate that the first peak in these patterns represents a true component.

TABLE IV
CONCENTRATION OF THE VARIOUS PROTEIN SOLUTIONS REPORTED IN FIG. 4

Flour sample	Protein concentration of solutions			
	First peak	Other peaks	Total	By Kjeldahl
Comanche	% 0.05	% 0.18	% 0.23	% 0.26
Chiefkan	0.05	0.20	0.25	0.25
E. Triumph	0.04	0.18	0.22	0.26
E. Blackhull	0.06	0.16	0.22	0.25

The electrophoresis patterns of the water-soluble protein of flour presented in Fig. 5 show that the patterns for all flours were alike. There was one main component of very low mobility (see Table V) and two minor components which had a higher mobility than the

TABLE V
ELECTROPHORETIC MOBILITIES OF PROTEIN COMPONENTS IN WATER EXTRACTS OF VARIOUS FLOURS, pH 2.15 (CM./SEC./VOLTS/CM. $\times 10^6$)

Chiefkan (Cj)		Early Blackhull (Bj)		Comanche (Hj)	
Ascend. boundary	Descend. boundary	Ascend. boundary	Descend. boundary	Ascend. boundary	Descend. boundary
0.69	0.67	0.03	0.06	0.01	0.07
4.06	3.58	3.14	2.97	2.34	2.19
10.35	10.38	9.84	9.46	9.81	9.42

major component. The mobilities of the protein components from Chiefkan flour were slightly higher than those for the other two samples. The outstanding difference was for the main component. There was a tendency for the slower moving of the two minor components of the Comanche sample to separate into two peaks; otherwise, the patterns for water-soluble protein did not vary from one flour sample to another. The patterns for each boundary are symmetrical for all determinations made at pH 2.15. However, when Early Blackhull was run at pH 9.72 the boundaries were not symmetrical, indicating a need for a more detailed study of the various protein fractions in buffers of several pH's.

Schwert, Putnam, and Briggs (9) give a concise discussion of component interaction in electrophoretic work and point out that at least two types of interactions between protein components may be expected. When interaction between components occurs the patterns are not symmetrical with respect to the number or the relative areas under the peaks, and the mobilities of one or more peaks in each boundary will vary from that characteristic of any component. Certain types of interaction may be weakened by increasing the ionic strength of the buffer with the results that the patterns become more symmetrical.

There was little evidence of component interaction in the samples run at pH 10.2 and the higher ionic strength, since the patterns shown in Fig. 2 were nearly symmetrical in both legs of the cell. When the ion strength was reduced, the pattern became less symmetrical (Fig. 3), indicating a tendency toward component interaction.

When the electrophoretic analysis was made in acidic buffers of pH 2.15 and an ionic strength of 0.03 there was marked asymmetry in the two boundaries, indicating very decided component interaction. Since this investigation was a comparative study of the proteins of different varieties of wheat, no attempt was made to study the type of interaction taking place or to reduce the component interaction by changing the ion strength over this pH range.

The patterns presented in Fig. 4 show that the proteins from all flours studied apparently undergo the same type of interactions because all ascending boundaries are similar and all descending boundaries are similar.

Since the electrophoresis investigation failed to detect differences in glutens from Chiefkan, Early Blackhull, and Comanche flours, it was decided to test the "bread-improving" power of each of these glutens when added to a common flour. Three flour mixtures were prepared to be used as substrata to test the glutens. Mixture No. 1 contained 9.1% protein, No. 2 contained 11.1% protein, and No. 3 contained 13.1% protein, all on a 14% moisture basis.

Two samples of gluten were prepared from each of the Chiefkan, Early Blackhull, and Comanche samples from Protection. One was prepared by washing the gluten from the flour in 0.15% salt solution, drying and grinding to pass a 10XX bolting cloth. The other was prepared by purifying the gluten, as suggested by Baker *et al.* (2), before drying and grinding.

Each mixture was baked with given quantities of the finely ground gluten added. When the protein content of the mixture was increased 0.6% by adding dried gluten flours there was very little difference in the response of any mixture to the purified and unpurified glutens.

The Chiefkan gluten (Cp) gave the least improvement in the loaf volume and the Comanche (Hp) gluten gave the greatest improvement, although the difference in the two was probably not significant.

When the protein content of the mixture was increased by approximately 1.2% by the addition of dried purified finely ground gluten, the response of each mixture to the added gluten was in the same order as reported above. However, when the increase of 1.2% was made by adding freshly washed, unpurified wet gluten to the mixtures, the Chiefkan gluten produced the greatest response. Furthermore, the response to wet gluten was significantly higher (above 1% level) than the response to dry gluten even though the protein content was increased by the same amount in each instance. The results for wet and dried gluten are compared in Table VI.

TABLE VI
PROTEIN CONTENT (14% MOISTURE) AND LOAF VOLUME OF BREAD
FROM FLOUR MIXTURES WITH ADDED GLUTEN

Sample	Gluten	Dry gluten		Wet gluten	
		% Protein	Loaf volume	% Protein	Loaf volume
Mixture 3	None	13.1	453	13.1	453
	Cp	14.5	573	14.2	654
	Bp	14.5	605	14.4	620
	Hp	14.4	587	14.4	634
Mixture 2	None	11.1	550	11.1	550
	Cp	12.7	609	12.7	615
	Bp	12.4	600	12.6	595
	Hp	12.4	621	12.8	617
Mixture 1	None	—	505	—	505
	Cp	—	523	—	641
	Bp	—	545	—	623
	Hp	—	550	—	615

In the light of the work reported here, which appears to agree with the work of Schwert *et al.* (9) who found no difference in the electrophoretic pattern of four gliadin samples regardless of the source of gliadin or method of preparation, it can be definitely stated that under the conditions of this investigation, the electrophoresis technique did not detect any significant differences in the proteins of the various flour samples. However, it should be emphasized that this does not mean it has been definitely established that differences do not exist. A more detailed study involving fractionation of the gluten into various protein fractions and improved solution techniques and buffer systems might detect small, but important, differences which were not

shown in the present investigation. However, the limited amount of electrophoresis data available on wheat proteins indicates that either there is virtually no difference in the composition of the gluten protein from poor quality and good quality flour, or the development and manipulation of the gluten during the sample preparation alters the protein in such a manner as to destroy any existing differences.

The baking data presented in Table VI appear to support this conclusion and indicate that the state of gluten development may be more responsible for flour quality than the chemical or amino acid composition of the gluten complex. This would agree completely with the electrophoretic work which failed to detect significant differences in the proteins from the various flour samples investigated.

Acknowledgment

The authors are indebted to Mrs. Elsie Leidheiser for assistance in preparing the gluten samples and making the baking tests.

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AIR, WATER VAPOR, AND CARBON DIOXIDE AS LEAVENING GASES IN CAKES MADE WITH DIFFERENT TYPES OF FATS¹

MAUDE PYE HOOD² and BELLE LOWE³

ABSTRACT

The relative increase in cake volume over batter volume attributable to air, water vapor, and carbon dioxide was investigated. The major increase in cake volume was produced by carbon dioxide, followed by water vapor and air, in the order given. The effectiveness of water vapor in the presence of air in leavening cakes varied with the type of fat used, being greatest with oil, intermediate with butter, and least with hydrogenated lard. Conversely, the effectiveness of carbon dioxide was greatest in the hydrogenated lard and least in the oil cake.

Air-evacuated batters showed very little increase in cake volume, indicating that the effectiveness of water vapor as a leavening agent depended on the presence and distribution of air in the batter. The cakes from the air-evacuated batters were considered unpalatable, whereas those leavened by air and water vapor and by carbon dioxide, air, and water vapor were acceptable.

Viscosity of the batters was affected by the mobility of the fats used and by the incorporation of gas in the batter. It was not always a good criterion of cake quality.

This investigation was undertaken to determine the proportionate leavening attributable to air, water vapor, and carbon dioxide in plain cake. Three fats were used to study the relationship, if any, existing between the type of fat and the leavening power of the three gases.

Good aeration of cake batter has long been considered of paramount importance in the production of light, good-quality cakes. Dunn and White (4) have reported that steam cannot materially increase the volume of cake during baking unless air pockets are present into which the steam may vaporize. They estimated that approximately half of the increase in volume of pound cake was due to thermal expansion of air. In their calculation, however, the initial quantity of air incorporated in the batter was included in the volume from air expansion. When air in their cakes was completely exhausted the resulting batter was described as a "custard-like cream," and there was no volume increase during baking. They found that the occlusion of a very small amount of air in the batter resulted in a definite increase in cake volume. Results, when an evacuated batter was remixed to incorporate air, showed a reasonably good cake but with decided evidence of overmixing.

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Barmore (1) showed that of the 1,120- to 1,620-ml. increase in volume during baking of angel cakes, only 350 ml. could be attributed to the expansion of air, the remainder to steam. He postulated that the water vapor causing the expansion came from the sides and bottom of the cake. Hence, the steam that leavened the cake must pass through the interior of the cake to escape.

Carlin (2) observed that very few, if any, new gas cells were formed when baking powder was added to the cake formula. The carbon dioxide evolved seemed only to enlarge the air cells already present and not to form new cells. During baking the fat melted and the air spaces moved into the flour-water phase, apparently moving in a definite convection pattern until near the end of the baking when the movement was described as being "violent and without direction."

It was recognized that changes in type or proportion of ingredients, may necessitate changes in the method or extent of combining ingredients, to obtain a satisfactory cake. Hence, the method selected for combining ingredients was one, which over several years in this laboratory, had produced good quality cakes with the three types of fats selected for use in this study.

Materials and Methods

An oil,⁴ butter, and a hydrogenated lard⁵ were the fats selected for use. The butter was made by the Dairy Industry Department of the College from sweet cream. It had about 2.25% salt content with a current score of AA. It was used as obtained. A series of cakes (24 per series) was made with each fat. Each series of cake batters was further subdivided into three portions, in which different leavening agents were used, i.e., (1) air and water vapor, (2) carbon dioxide, air, and water vapor, and (3) water vapor alone.

The following formula was used:

Ingredients	Grams	Grams per 100 g. of flour
Fat	122	43.0
Salt	.3	1.0
Sugar	150	52.0
Flour, cake	284	—
Milk, whole liquid	244	85.0
Egg-magma meringue:		
Whole liquid egg	96	33.8
Sugar	150	52.0

Baking powder (2.75 g./100 g. flour) was used in only one-third of the batter from each mix. A sulfate-phosphate type was chosen because it released only a small amount of carbon dioxide at room temperature.

⁴ Wesson oil, The Southern Oil Company.

⁵ Clix, Cudahy and Company.

All ingredients, except the milk and eggs, were incubated at $26^{\circ}\text{C.} \pm 1^{\circ}\text{C.}$ Milk and egg were brought to 25°C. just prior to combining the ingredients. The work was conducted in a small laboratory in which the temperature was held at $25.3^{\circ}\text{C.} \pm 2.5^{\circ}\text{C.}$

Method of Combining Ingredients. The fat and salt were creamed on speed two of the Kitchen Aid mixer (Model G) for 30 seconds. Then 150 g. of sugar were added gradually during a 4-minute period. Creaming was continued for 10 minutes.

Approximately one-sixteenth of the flour was added to the creamed mixture with 20 strokes, using a hand balloon whip. About one-fifth of the milk was then added with 20 strokes. This procedure was repeated. Next one-half of the remaining flour and milk was added with 80 strokes. The remainder of the milk and flour was blended with the batter with 90 stirs of the hand whip.

The whole egg was beaten on speed three for approximately 1.5 minutes. Then the speed was reduced to two and the sugar added gradually while beating was continued for 4.5 minutes. The mixture was scraped down from the sides of the bowl, then beaten 30 seconds. The egg-magma meringue was folded into the batter with 25 strokes.

Division of the Batter. At expedient stages of mixing, the batter was divided into three portions. One-third of the creamed mixture was removed after creaming was completed for the cake to be leavened by air, water vapor, and carbon dioxide. To this, one-third of the flour (previously sifted with the baking powder), one-third of the milk, and one-third of the egg-magma meringue were added. The remaining batter was divided into two portions after the flour, milk, and egg had been added. The air was removed from one portion of the batter by means of a water vacuum pump. Obviously some water was also removed but the fluidity of the batter indicated that enough water remained to furnish leavening by water vapor. The third portion of the batter was used for the cake leavened by air and water vapor.

Baking. The cakes were baked in a thermostatically controlled gas oven maintained at 185°C.

Calculations. Data were taken to study the effect of type of fat and of leavening gases on certain properties of the batters and cakes, and to compute the leavening power of each gas. The specific gravity (3) of the batter was computed from the weight of a measured volume of batter in milliliters. The linespread of Gravemeyer and Pfund (5) was used to determine the consistency of the batters. Cake volumes were determined by seed displacement.

For air and water vapor leavened cakes:

$$\text{Air factor} = \frac{\text{Temperature of batter as it goes in oven} + 273}{\text{Temperature of cake as it comes from oven} + 273} - 1$$

$$\text{Specific volume of batter} = \frac{1}{\text{Specific gravity of batter}}$$

$$\text{Volume of weighed batter in pan} = \text{Specific volume of batter} \times \text{weight of batter}$$

$$\text{Total increase in volume of cake} = \text{Measured volume of cake} - \text{volume of batter}$$

$$\text{Volume of weighed air-evacuated batter} = \text{Specific volume of air-evacuated batter} \times \text{weight of batter}$$

$$\text{Volume of air in batter} = \text{Volume of batter with air} - \text{volume of air-evacuated batter}$$

$$\text{Volume of heated air} = \text{Air factor} \times \text{volume of air in batter}$$

$$\text{Volume increase from air expansion} = \text{Volume of heated air} - \text{volume of air in batter}$$

$$\% \text{ volume increase from air expansion}$$

$$= \frac{\text{Increase from air expansion} \times 100}{\text{Total increase in volume}}$$

$$\text{Total increase in cake volume}$$

$$= \frac{\text{Total volume increase at end of baking} \times 100}{\text{Volume of weighed batter}}$$

For air, water vapor, and carbon dioxide leavened batters:

$$\text{Volume increase from carbon dioxide} = \text{Total volume increase of cake} - \text{total volume increase of air and water vapor leavened cake}$$

Compute percentages as above.

Palatability. The cakes were rated for grain (30), tenderness (20), texture (smoothness or lack of harshness, 20), and eating quality (equivalent to flavor, if flavor is considered as a combination of aroma, taste, and tactile sensations, 30). The highest possible score for each factor is given in parentheses.

Results and Discussion

Appearance. Typical sections from the centers of the oil cakes are shown in Fig. 1. The air and water vapor leavened cakes, when removed from the oven, had straight sides close against the pan, but immediately shrank until the sides pulled away from the pans and were slightly concave in contour. The crumb of the cake was velvety, tender, moist, and of fine uniform grain, but the top crust did not brown. There was a tendency towards compactness and small soggy spots in some of the cakes.

When baking powder was included in the formula to furnish carbon dioxide in addition to air and water vapor for leavening, the cakes were lighter, less moist, and evenly browned. The cakes had straighter sides than the cakes leavened with air and water vapor. The grain was open and loose, sometimes coarse, and the crumb had a tendency to be harsh and crumbly. Cakes made with oil showed more evidence of harshness and crumbliness than cakes made with the other fats.

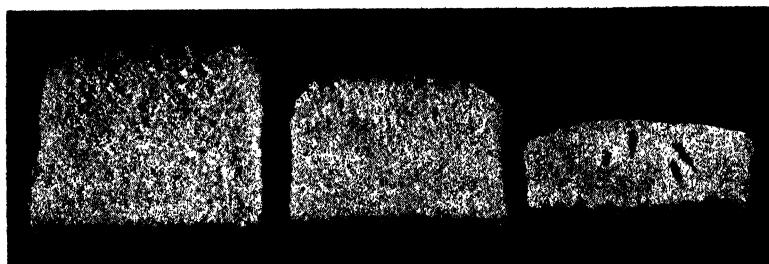


Fig. 1. Cakes containing oil leavened by: (left) carbon dioxide, air, and water vapor; (middle) air and water vapor; (right) water vapor (air-evacuated batter).

The cakes from air-evacuated batters, which were leavened by water vapor, had very small volumes, were distorted in shape, and evidenced little or no cell structure. The interior appeared more like a starch pudding than a cake.

TABLE I
MEAN PALATABILITY SCORES OF CAKES: GRAIN, TENDERNESS, TEXTURE,
EATING QUALITY (AROMA, TASTE, TACTILE SENSATION),
AND TOTAL SCORES

Fat used	Grain	Tenderness	Texture	Eating quality	Total score
AIR AND WATER VAPOR LEAVENED CAKES					
Butter	23.4	16.0	17.2	24.4	81.0
Oil	24.1	16.0	18.1	24.5	82.7
Hydrogenated lard	21.6	15.9	17.6	22.2	77.4
CARBON DIOXIDE, AIR, AND WATER VAPOR LEAVENED CAKES					
Butter	24.2	17.6	17.1	24.6	83.5
Oil	24.6	17.5	16.1	24.4	82.5
Hydrogenated lard	25.0	16.9	17.2	24.2	82.3
WATER VAPOR LEAVENED (AIR-EVACUATED) CAKES					
Butter	1.2	3.4	0.9	1.3	6.8
Oil	0.0	1.5	0.1	0.5	2.1
Hydrogenated lard	2.0	3.7	1.5	1.4	8.7

Palatability. Statistical analysis of the palatability scores indicated no significant differences among the total scores of cakes with different fats (Table I). There was no significant difference between the palatability scores of cakes leavened by air and water vapor and cakes leavened by carbon dioxide, air, and water vapor. Scores of cakes leavened by water vapor alone were extremely low and the cakes were described as unpalatable. Statistically, the total palatability scores of the water vapor leavened cakes were lower (the differences were highly significant) than total scores of cakes leavened with (1) air and water vapor or (2) carbon dioxide, air, and water vapor.

Average texture scores of cakes leavened by air and water vapor and of cakes leavened by carbon dioxide, air, and water vapor showed only slight differences, but the differences favored the cakes leavened by air and water vapor. Although the amount of baking powder used in this study was the lowest quantity found desirable in an earlier study in this laboratory, the cakes tended to have a coarse, crumbly, and harsh texture typical of too much baking powder. The texture scores of all cakes from air-evacuated batters averaged less than two.

Average grain ratings of cakes leavened by air and water vapor were always slightly lower than the corresponding cakes leavened by carbon dioxide, air, and water vapor, but decidedly higher than ratings of cakes from the air-evacuated batters.

Average eating quality scores were similar for the two groups of cake leavened by air and water and by carbon dioxide, air, and water vapor. In contrast, the flavor scores of the cakes leavened by water vapor were extremely low; these cakes were considered unpalatable (Table I).

Cake Volumes. A striking difference was evident in cake volumes obtained with different leavening agents (Figs. 1 and 2 and Table II). Volumes of cakes leavened by carbon dioxide, air, and water vapor were always largest; those leavened by air and water vapor were intermediate; and those leavened by water vapor alone were the smallest. These differences were highly significant statistically. Comparison of the individual fats showed a highly significant difference between the mean volumes of oil cakes (423.4 ml.) and butter cakes (388.7 ml.), a significant difference between means of oil cakes and hydrogenated lard cakes (402.1 ml.), but no significant difference between hydrogenated lard and butter cakes.

Leavening Attributed to Air. The computations for leavening by air have been based on the assumption that the air incorporated in the batter was 100% effective as a leavening agent. Such was not the case. The maximum expansion of the batter was not the same as the measured volume of the cake (taken when cooled). Maximum ex-

pansion of the batter usually occurred just before baking was completed. Cakes usually shrank some before removal from the oven and continued to shrink after removal. Since the volume increase attributed to air was the maximum expansion of which the air was capable, obviously the proportion of the volume increase attributed to air of the measured cake volume was larger than actually occurred. In addition, no account was taken of the air and water vapor lost during baking. Nevertheless, the results do represent the maximum amount of increase of which the air is capable.

TABLE II

MEANS OF MEASURED CAKE VOLUME, VOLUME INCREASE OVER THE
INITIAL BATTER VOLUME, SPECIFIC GRAVITY, AND
VISCOSITY AS LINESPREAD

Cakes	Cake volume, ml.	Total volume increase over initial volume, ml.	Specific gravity	Viscosity (linespread)
BATTERS CONTAINING AIR				
Butter	388.1	88.1	0.80	0.9
Oil	417.5	142.3	0.81	4.7
Hydrogenated lard	366.8	66.8	0.80	0.6
BATTERS CONTAINING AIR AND BAKING POWDER (YIELDING SOME CO ₂)				
Butter	514.5	216.8	0.79	0.9
Oil	586.2	286.7	0.77	4.0
Hydrogenated lard	565.0	247.5	0.80	0.5
AIR-EVACUATED BATTERS				
Butter	256.8	24.0	1.03	5.0
Oil	256.8	24.0	1.03	7.2
Hydrogenated lard	291.8	61.6	1.02	2.8

The increases in cake volume attributable to thermal expansion of the occluded air were 19.8, 11.4, and 25.0% for cakes containing butter, oil, and hydrogenated lard, respectively, when they were leavened with air and water vapor; the corresponding volume increases for cakes leavened with carbon dioxide, air, and water vapor were 8.0, 5.6, and 6.7%. The volume increase attributable to air expansion measured in milliliters was nearly the same in all groups of cakes in which air was a leavening agent. In the same order as the percentage increase, the increase in milliliters was 17.4, 16.1, and 16.7, and 17.3, 16.0, and 16.6. This indicates that all cakes had very nearly the same amount of air incorporated in the initial batter. The similarity of the

specific gravities of the batters from the three types of fats (Table II) also supports this view.

If the occluded air in the initial batters had been considered as a part of the cake volume increase, thus using the air-evacuated batter as a basis for computing increase in cake volume attributed to air, then the values would have been 54.5, 38.3, and 66.3% for butter, oil, and hydrogenated lard, respectively. These percentages are computed in the same manner as those of Dunn and White (4) and are more nearly in keeping with the approximate 50% increase in pound cake volume attributed to air by these investigators.

Leavening Attributed to Water Vapor. The remainder of the volume increase of cakes leavened by air and water vapor, after deduction of the volume increase brought about by the thermal expansion of the air, was attributed to water vapor. Since the leavening attributed to air was approximately the same in all cakes of the air and water vapor group, the wide variance in total volume was produced by water vapor, Fig. 2. It seems, therefore, that the effectiveness of water vapor in leavening the cakes varied with the type of fat used. The volume increase brought about by water vapor for cakes with butter, oil, and hydrogenated lard was: (1) for the air and water vapor leavened group 70.7, 126.2, and 50.1, (2) for the carbon dioxide, air, and water vapor leavened cakes 70.1, 126.4, and 50.0, and (3) for cakes leavened by water vapor alone 24.0, 24.0, and 61.6 milliliters. Thus the volume increase, with one exception, was always greatest in the cakes containing oil and least in the hydrogenated lard cakes. The one exception was in the group of water vapor leavened cakes where the increases for butter and oil were the same. The volume increases brought about by water vapor are particularly interesting when contrasted with the volume increases attributed to carbon dioxide.

When water vapor was the only leavening agent the volume increase of the butter and oil cakes was very small. Under the vacuum the batter crept up the side of the cell and had a honeycombed structure. Upon removal of the vacuum, the batter collapsed.

Some of the air-evacuated batters of this study showed no increase in volume during baking, but those in which some air remained (usually those of the hydrogenated lard series, but also including the oil cake shown in Fig. 1) had definite volume increases in baking. Throughout the experimental period difficulty was encountered in removing the air from batters containing hydrogenated lard. The hydrogenated lard had a much higher melting point than butter, and this resulted in batters of high viscosity which probably accounted for at least a part of the difficulty in removing the air from them. Whatever the explanation, the hydrogenated lard batters held their air ten-

aciously and on one occasion the final volume of the cake was within 30 ml. of the corresponding cake of the air and water vapor leavened group. Dunn and White (4) reported no increase in the volume of the air-evacuated pound cake batter of their study, but a decided increase (with poor structure) when only a small amount of air was occluded in the batter.

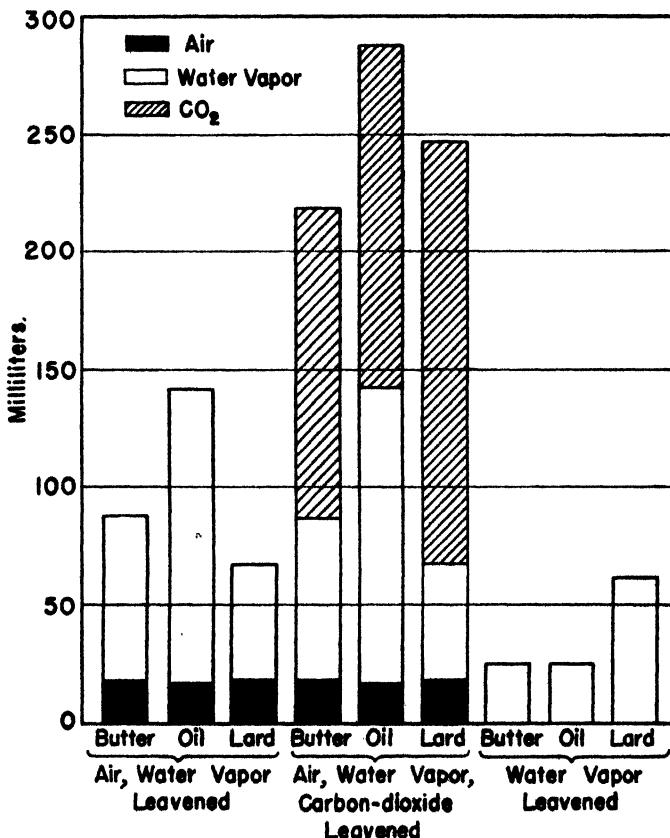


Fig. 2. Cakes made with butter, oil, and hydrogenated lard; leavened by (1) air and water vapor, (2) carbon dioxide, air, and water vapor, and (3) water vapor. The total volume increase over the initial volume of the batter and the proportion of the increase attributed to carbon dioxide, air, and water vapor are shown.

Moisture that was condensed in the pans beneath the water vapor leavened cakes, when the cold cakes were removed from the pans, gave evidence that more vapor had been formed than could escape, either around or through the cake. This could be interpreted as indicating that air spaces are necessary for the vaporization and diffusion of water vapor in the batter.

Leavening Attributed to Carbon Dioxide. After the total cake volume increase above initial volume of the batter was computed, the percentage increase attributed to carbon dioxide was derived by subtracting the total increase of the corresponding air and water vapor leavened cakes. Although this could not be taken as an exact measurement, it seemed a logical relative determination, since the batters and baking conditions were identical except for the addition of baking powder to the carbon dioxide, air, and water vapor leavened cake batters.

The average increases in cake volume attributed to carbon dioxide were 59.7, 50.2, and 73.1% for butter, oil, and hydrogenated lard, respectively. Here again a difference in the effectiveness of a leavening agent is exhibited with the type of fat used. The percentage volume increases with carbon dioxide, in contrast with the volume increase (per cent) attributed to water vapor, were greatest with the firmest fat, the hydrogenated lard, and least with the mobile fat, the oil.

Although the thermal expansion of the air accounted for a relatively small percentage of the volume increase in the cakes, the air incorporated in the batters apparently formed the cell structure into which the water vapor could easily escape. It was interesting that little or no volume increase occurred in the air-evacuated batters, when water vapor was the only leavening agent. This indicates an interdependence among the leavening gases and shows that aeration of cake batters has a function beyond that of increasing cake volume. The presence of air is necessary before the water vapor can function as a leavening gas. These results confirm those of Dunn and White (4) and of Barnmore (1). A still greater interdependence among the gases is suggested by Carlin (2). He found that carbon dioxide, in the early stages of baking, does not form new gas cells but expands the existing air bubbles.

Viscosity as Affected by Fats. References are often made in the literature to viscosity of cake batters as an index of cake quality and cake volume. An opportunity was offered in this study to examine the influence on batter viscosity of (1) the mobility of the fat used and (2) the incorporation of gas.

Decided viscosity differences, as measured by linespread, occurred among batters containing different fats. As would be expected from the physical condition of the fats, oil batters showed the greatest fluidity (4.0 to 7.2), butter batters were intermediate (0.9 to 5.0), and hydrogenated lard batters least (0.5 to 2.8). In linespread determinations the batter having the least viscosity covers the greatest surface area of concentric rings one-eighth inch apart. Statistical analysis of the data showed highly significant differences between the viscosity

of oil batters and either of the batters containing the other two fats, and a significant difference between butter and hydrogenated lard.

Viscosity as Affected by Leavening Agents. The batters, on the basis of gas in the batter, comprised three groups, those containing (1) air, (2) air and some carbon dioxide, and (3) the air-evacuated batters. The viscosity differences among these batters were significant, largely because of the removal of air in the third group.

The viscosity means of the batters containing air were of the same order as the viscosity of batters with the different fats. Hydrogenated lard was greatest with 0.6, butter intermediate with 0.9, and oil fell considerably lower with a mean of 4.7. As might be expected, the viscosity means of batters containing carbon dioxide and air (since only a small portion of the baking powder would react to form carbon dioxide at the batter temperature) varied very little from the means of the batters containing only air (Table II).

The viscosity of the air-evacuated batters with all fats was much lower than that of batters containing either air or air and carbon dioxide and made with corresponding fats. This indicates the influence of incorporated gas on batter viscosity, whereas the difference still existing among the batters containing different fats represents the effect of the fat itself on batter viscosity. The order of viscosity means of the air-evacuated batters was hydrogenated lard 2.8, butter 5.0, and oil 7.2.

Thus, under the conditions of this study both the incorporation of gas and the viscosity of the fat had significant effects on the viscosity of the batter. In addition, viscosity was not always a good criterion of cake quality.

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EFFECT OF SYNTHETIC PLANT GROWTH STIMULANTS ON SOME PROPERTIES OF CEREAL PROTEINS AND ON MIXOGRAM PATTERNS¹

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ABSTRACT

The concentration of alpha-sodium naphthyl acetate and sodium phenyl acetate was directly related to the per cent of total protein extracted from rye, wheat, barley, and millet flour, the percentage extracted decreasing in the order in which the cereals are named. The highest concentration of growth stimulant employed was 15.0%, at which point the effect of concentration on per cent of protein extracted appeared to be approaching a constant. The effect of different stimulants on protein solubility varied; sodium naphthyl acetate had the greatest effect. This substance in relatively low concentration apparently tended to coagulate wheat flour protein dispersions in water, while at higher concentrations it increased the solubility of this protein. Sodium naphthyl acetate and 2-4 dichloro-phenoxy sodium acetate were very effective precipitating agents for 0.1 N acetic acid dispersions of gluten, removing approximately 98.0% of dispersed protein at a concentration of 1400 mg. per 100 ml. of dispersion and greatly decreasing the relative viscosity. From the limited data collected, the final effect on solubility appeared to be independent of the individual stimulant employed.

The initial influence of stimulants on mixogram pattern of hard red spring wheat flours was to increase the strength of the curve; but beyond a concentration of approximately 2.0% of weight of flour used, a deleterious effect which increased with addition of stimulant was noticeable. Different wheat varieties were affected in the same manner.

It is apparent that synthetic plant growth stimulants have a marked effect on some properties of cereal proteins, and that this effect varies with the stimulant.

A large number of organic chemicals is known to promote the growth of plants. These substances contain either an unsaturated or an aromatic ring, and a carboxyl (or a group readily converted by the plant to a carboxyl) separated from the ring by at least one carbon or oxygen atom. Three naturally occurring substances have been isolated which promote growth. These are auxentriolic acid (auxin a), auxenolonic acid (auxin b), and indoleacetic acid (9). Not only do these materials promote growth, but under certain conditions they may inhibit growth, leading to their use in destroying weeds.

Grace (2) found that 5.0% neutral sodium salt solutions of the group of chemicals comprising synthetic plant growth stimulants dispersed undenatured wheat gluten. Sodium naphthyl acetate ap-

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peared to be the most efficient, dispersing essentially as large a proportion of the gluten as sodium hydroxide and 10.0% sodium salicylate. Grace noted that this property might be related to the mechanism of plant responses to these compounds. No attempt was made to determine the effect of growth stimulant concentration on quantity of gluten extracted.

The present work consists of a study of the effect of plant growth stimulants on the quantity and properties of protein dispersed from several cereals, as well as the influence of stimulant on the mixogram pattern of hard red spring wheat flour.

Materials and Methods

The flours employed in this study were approximately 95.0% long patents which had been experimentally milled from hard red spring wheat varieties grown on experimental plots in 1946. The varieties were Thatcher, Pilot, Mida, and Cadet, each grown at branch experiment stations located at Dickinson, Langdon, and Edgeley. These included a range of protein from 13.2 to 15.4% and comprised three mixogram types, strong, medium, and weak. A composite sample was prepared from a blend of several commercially important hard red spring wheat varieties grown at several points in North Dakota. The barley variety was Manchuria (12.5% protein) while the rye was Washington Imperial (7.2% protein), both grown in the Experiment Station plots at Fargo in 1945. The millet was a yellow variety, Proso (8.6% protein), purchased from a grocery store. These three protein values ($N \times 6.25$) are expressed on a 13.5% moisture basis. All samples were sound and free from damage.

The wheats were milled by the method described by Sibbitt, Scott, and Harris (8) for the Allis mill, while the other three cereals were milled by suitable modifications of this procedure. The millroom was maintained at approximately 70°F. and 65% relative humidity.

The effect of growth stimulants on protein solubility was determined employing 5 g. of flour in 100 ml. of distilled water containing the required concentration of stimulant and agitating gently for 6 hours in a rotary shaker (4). The flour suspensions were centrifuged and the protein content of the decanted solution determined by the Kjeldahl-Gunning method. When examining the coagulating action of growth stimulants, suitable additions of the active substance were made to the flour suspensions at the beginning of shaking. Six hours, with gentle shaking, was allowed for the stimulants to act on the flour protein. The dispersions were then centrifuged, and the nitrogen content and viscosity of the supernatant liquid determined. Viscosity determinations were made with the Ostwald pipette at 25°C. \pm 0.05°C.

No effort was made to standardize the pipette since it has been shown that relative rather than absolute viscosity values are of major importance in biological chemistry (1, 7). The pH of the dispersions was obtained by a Beckmann pH meter.

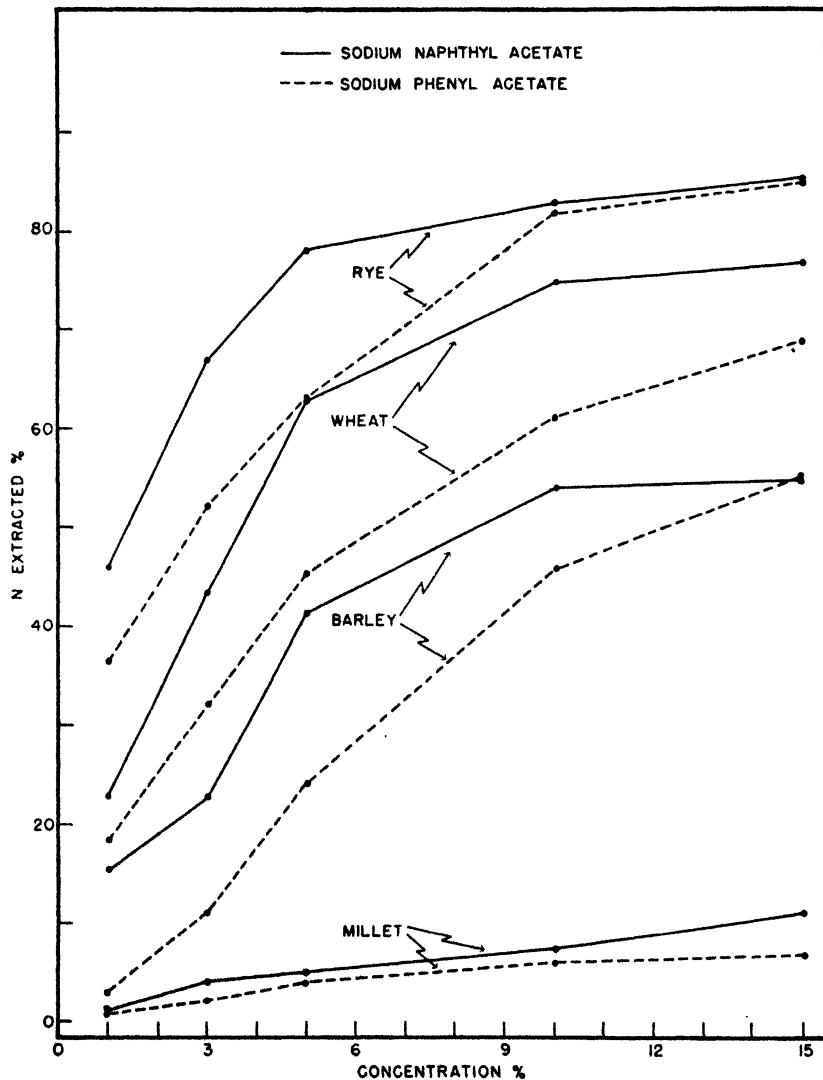


Fig. 1. Solubility of four cereal flour proteins in various concentrations of sodium naphthyl and sodium phenyl acetates.

The gluten dispersions were made by the method described by Harris and Johnson (5) using the Waring Blender and 0.1 *N* acetic acid. After removal of undispersed material by centrifuging, suitable

aliquots were taken for protein precipitations. The dry salt was added to the dispersion.

The mixograms and their dimensions were obtained by the procedure described by Harris (3) using flour and distilled water at the absorption found when baking the flours. The stimulant in the dry form of its sodium salt was added to the water immediately before mixing. Suitable concentrations were selected from preliminary determinations.

Results

The results secured from the extraction and viscosity determinations are presented, with one exception, in the form of curves, with the actual values shown as dots in the figures. This mode of presentation facilitates comparisons, and emphasizes the fact that precise values cannot be expected in this type of investigation. A table of mixogram dimensions is included to enable comparisons to be made among the different treatments with growth stimulants.

TABLE I

COMPARATIVE PROPORTIONS OF THE TOTAL NITROGEN EXTRACTED FROM CEREAL FLOURS BY DIFFERENT REAGENTS

Extractant ¹	Nitrogen extracted			
	Rye	Wheat	Barley	Millet
Sodium salicylate, 10.0%	%	%	%	%
2-4 Dichloro-phenoxy sodium acetate, 1.1%	77.0	78.7	56.4	12.7
2 Naphthoxy sodium acetate, 1.3%	50.7	49.7	19.7	8.0
2-4-5 Trichloro-phenoxy sodium acetate, 0.6%	40.0	35.0	16.7	7.3
Distilled water	38.0	16.0	15.4	2.6
	37.0	15.8	15.6	3.0

¹ Growth stimulant solutions were all saturated.

Note: pH of extracts was 6.8.

Effect of Plant Growth Stimulants on Protein Solubility. The results secured from treating the four cereal flours with various concentrations of sodium naphthyl acetate and sodium phenyl acetate are shown in Fig. 1. As noted by Grace (2) the former compound consistently extracts more protein than the phenyl salt. The concentration-extraction curves begin to level off after 5.0% concentration for sodium naphthyl acetate, and after 10.0% for sodium phenyl acetate. Rye protein was most easily extracted by both substances, with wheat second, and barley third. The proportion of protein removed from millet was very small. At the higher solvent concentrations there appeared to be some tendency for the curves to converge. Grace used the 5.0% level, where the curves are more divergent, and differences

between the two stimulants are most marked. The pH of all dispersions was approximately 6.8.

In Table I are data showing the per cent of nitrogen extracted from the flours by various substances, including three growth stimulants.

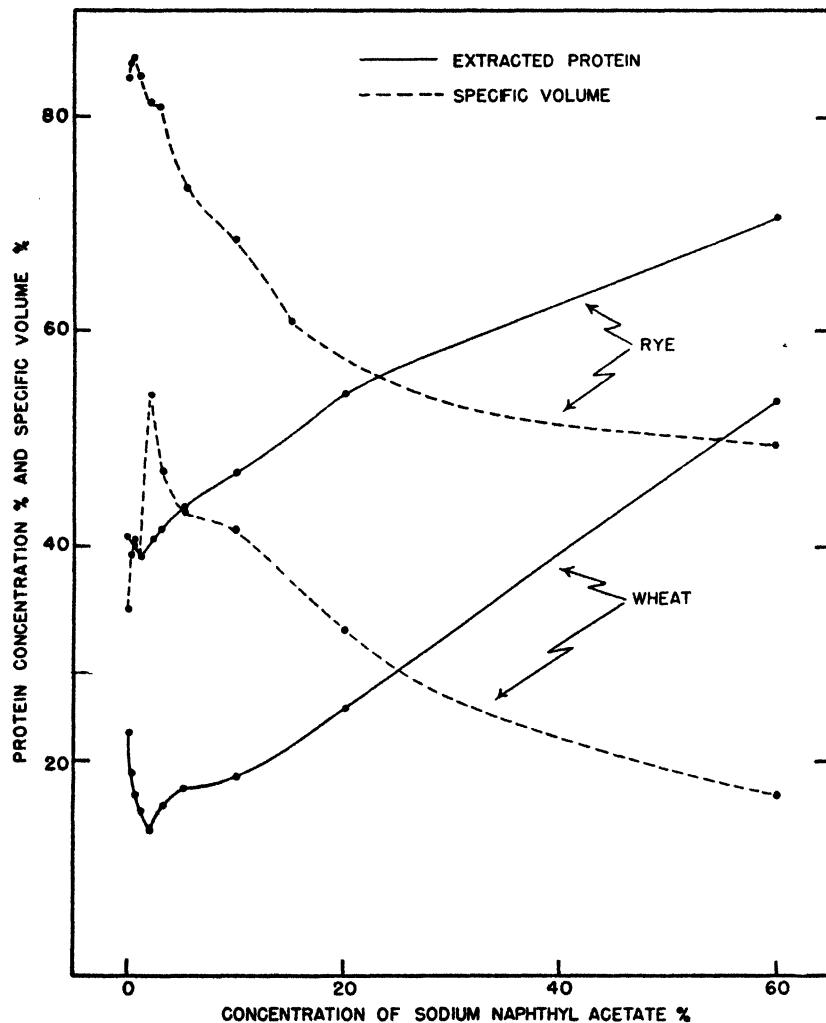


Fig. 2. Effect of concentration of sodium naphthyl acetate on protein extraction and specific volume of the protein micelle. Concentration of stimulant expressed as per cent of flour.

The concentrations are different in each solution because of the varying solubilities of the hormones, so direct comparisons cannot be made in regard to the relative solvent power. The relative insolubility of the proteins of millet is very marked, only 3.0% of the total nitrogen being

extracted by distilled water, while rye has 37.0% removed by this solvent.

Fig. 2 shows the effect of various concentrations of sodium naphthyl acetate solution on the amount of protein extracted from wheat and rye flours, as well as the influence on the specific volume of the protein particle in dispersion (6). This latter effect was investigated because it was found that the growth stimulants induced remarkable changes in the mixogram pattern of bread wheat flours (Fig. 6). At low initial concentrations there is a decrease in the amount of protein extracted for both wheat and rye, followed by a regular increase after a con-

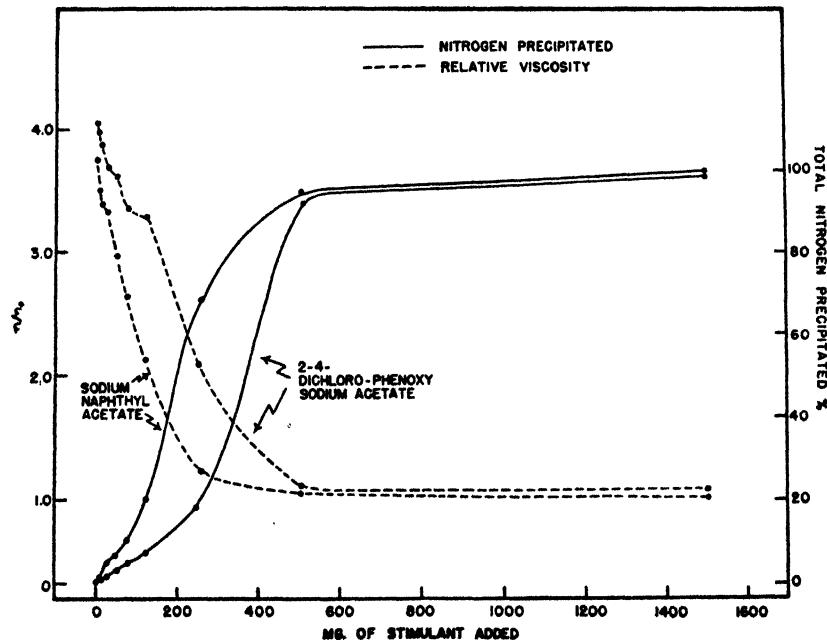


Fig. 3. Relations between stimulant concentration and per cent of total dispersed protein precipitated from gluten dispersions in 0.1 N acetic acid. Effect of stimulant on the relative viscosity, η/η_0 , is also shown.

centration of 0.25% was attained. Specific volume is affected in the opposite direction: first there is an increase in particle size as coagulation proceeds, and solubility decreases, while later the particle size progressively decreases as solubility rises. The initial effect on particle size is quite marked for wheat. From these results it is concluded that the initial effect of stimulants is to depress solubility by a coagulative influence, followed by a dispersive action at higher dosages.

Effect of Plant Growth Stimulants as Protein Precipitating Agents. Fig. 3 shows the results secured from additions of sodium naphthyl acetate and sodium dichloro-phenoxy acetate to dispersions of gluten

protein in 0.1 *N* acetic acid. The stimulants cause increasing precipitation of protein from the dispersion until a dosage of 500 mg. per 100 ml. is reached, after which further increase in reagent concentration has little influence on the quantity of protein removed. Sodium naphthyl

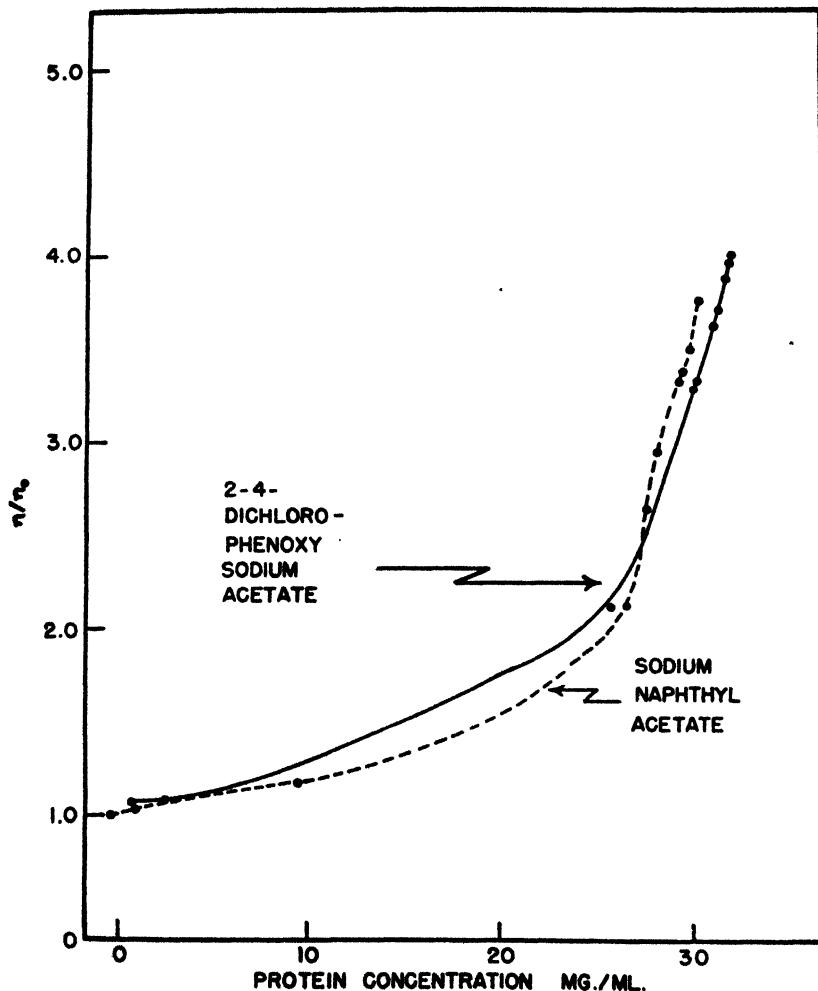


Fig. 4. Relation between relative viscosity and protein concentration in gluten dispersions in 0.1 *N* acetic acid. Data secured by precipitating the protein by graduated additions of two growth stimulants (Fig. 3).

acetate has a slightly greater effect than sodium dichlorophenoxy acetate until the maximum is reached, when the two salts become practically identical in their effects. The effect of the growth stimulants on relative viscosity is approximately the same as on concentration, viscosity decreasing as stimulant concentration increases until a

limit is reached beyond which there is little further increase as more stimulant is added.

The relation between viscosity and protein concentration shown in Fig. 4 reveals a marked fall in viscosity as the quantity of protein in dispersion decreases. The rather sharp inflection in the viscosity curve at approximately 25 mg./ml. concentration of protein might indicate a change in the type of protein fraction precipitated. The "glutenin" fraction, which is thought to include the larger particles, would be present in the dispersion before much protein had been removed by the growth stimulant, and would tend to affect viscosity more than other protein fractions which consist of smaller particles. The curves for the two stimulants, sodium naphthyl acetate and 2-4 dichloro-phenoxy sodium acetate, are practically superimposed, and show the familiar curvilinear relationship between protein concentration and viscosity.

Effect of Plant Growth Stimulants on Mixogram Patterns. Fig. 5 shows the effect of several plant growth stimulants on the mixogram



Fig. 5. Effect of different synthetic plant growth stimulants on the mixogram pattern of a hard red spring wheat flour. Concentration expressed as per cent of flour. A, control; B, 2 naphthoxy sodium acetate, 0.78; C, 2-4-5 trichloro-phenoxy sodium acetate, 0.36; D, 2-4 dichloro-phenoxy sodium acetate, 0.66; E, sodium phenyl acetate, 5.0; F, sodium naphthyl acetate, 5.0.

pattern of a flour experimentally milled from a number of hard red spring wheat samples. 2 Naphthoxy sodium acetate, 2-4-5 trichloro-phenoxy sodium acetate, and 2-4 dichloro-phenoxy sodium acetate appeared to increase the strength of the curve in the order named. Sodium phenyl acetate and sodium naphthyl acetate, however, when present in 5.0% concentration, markedly reduced the strength of the mixogram. The concentration of the first three substances employed was low (Fig. 5), owing to their poor solubility in water. This difference in concentration will significantly affect the curve characteristics, and operates against making direct comparisons regarding the effects of the five growth stimulants. However, it was later found that treatments of sodium naphthyl acetate in the 0.5% to 1.0% concentration range (Fig. 6) gave somewhat similar curve patterns with the same flour. There is little doubt that increasing the concentration of the first three salts would also cause a reduction in the strength of the mixogram. These data indicate that plant growth stimulants strongly affect mixing requirements. The strength of the effect tends to be related to the physiological activity of the compound in conformity with *a priori* expectations.



Fig. 6. Relations between concentration, as per cent of flour, of sodium naphthyl acetate, and the mixogram patterns of three hard red spring wheat flours.

Fig. 6 represents the influence of different concentrations of the most active stimulant, sodium naphthyl acetate, on the mixogram pattern of three hard red spring wheat flours possessing quite different mixing properties. The wheat protein content of these samples was as follows: Thatcher, 14.9%; composite, 12.9%; and Pilot, 13.6%. The normal mixograms secured from these flours are shown at the left of the figure; the differences in pattern type are quite evident. The initial effect of the stimulant was to increase all four of the dimensions measured for the three flours. This increased strength persisted until a concentration of 2.0% was reached for Thatcher, and 1.0% for the composite flour and for Pilot, although there was very little difference between the 0.5% and 1.0% treatments for the latter. Beyond this point all curves progressively weakened as stimulant concentration increased. In view of the effect of these substances on protein solubility in aqueous solution it seems logical to assume that the initial increase in the strength of the curves is due to a coagulative effect on the protein, followed beyond the optimum point by a dispersive action which would tend to weaken the physical properties of protein and result in a decrease in the strength of the curve.

The average dimensions of four mixogram properties secured from flours representing four hard red spring wheat varieties grown at three North Dakota stations in 1946 are shown in Table II. These varieties cover the range to be found in mixogram properties among spring wheats; Cadet has probably the strongest pattern, with Thatcher second in strength, and the remaining two varieties weaker in type. One per cent of stimulant increases the value for all four properties for all the four wheats, the greatest effect being probably in dough development. The next treatment, 2.0%, slightly decreased dough development from the 1.0% values. For dough stability, there was a decided increase, while the other two dimensions were not greatly altered from the previous values. The highest concentration of stimulant, 5.0%, greatly reduced all mixogram properties, and yielded lower values than the control, though in some instances the latter differences were not large. All flours appeared to be affected in much the same manner, regardless of curve type.

The data show that the character of the mixogram may be markedly changed by the addition of plant growth stimulants. A flour with weak mixing properties may approach a strong type flour in behavior upon suitable treatment with these substances. Conversely, a flour with too strong mixing requirements could exhibit weaker characteristics by a heavier treatment. It is believed that the baking quality of flour could be greatly changed by this class of substances provided the

TABLE II

EFFECT OF SODIUM NAPHTHYL ACETATE ON THE MIXOGRAM DIMENSIONS OF FOUR HARD RED SPRING WHEAT FLOURS

Wheat variety	Conc. of stimulant ¹	Dough development	Dough stability	Curve height	Curve width
Thatcher	0	4.6	4.9	7.6	1.1
	1.0	8.7	8.0	7.7	1.6
	2.0	7.5	9.5	7.8	1.8
	5.0	3.9	3.9	6.8	0.9
Pilot	0	4.1	3.8	7.1	0.9
	1.0	6.0	4.6	7.8	1.1
	2.0	6.2	6.3	7.4	1.7
	5.0	2.9	3.1	6.3	0.6
Mida	0	3.5	3.5	7.1	0.9
	1.0	5.9	4.5	7.7	1.3
	2.0	5.2	6.5	7.5	1.6
	5.0	2.9	3.6	6.1	0.8
Cadet	0	5.7	6.3	6.8	1.1
	1.0	10.7	9.8	7.8	1.5
	2.0	10.2	12.0	7.6	1.7
	5.0	4.4	6.0	6.6	1.0
MEANS FOR ALL VARIETIES					
	0	4.5	4.6	7.2	1.0
	1.0	7.8	6.7	7.8	1.4
	2.0	7.3	8.6	7.6	1.7
	5.0	3.5	4.2	6.5	0.8

¹ Expressed as per cent of quantity of flour used (25 g. on 13.5% moisture basis).

lethal effect of the stimulant on the yeast and other enzyme systems could be eliminated.

The data show that synthetic plant growth stimulants exert a very marked effect on the proteins of wheat, rye, and barley. Some stimulants in aqueous solution are very efficient extractives for wheat gluten, but function as precipitating reagents for gluten dispersed in 0.1 *N* acetic acid. At relatively low concentrations, they act as coagulative agents for water-soluble protein, and at higher concentrations they perform as dispersive substances. Their effect as growth stimulants at concentrations very much below these employed here suggest that their principal action on plant growth is through enzyme systems, rather than on the plant proteins *per se*.

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A REVISED METHOD FOR THE DETERMINATION OF BROMATE IN BROMATED FLOURS¹

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ABSTRACT

The method of Hoffer and Alcock (1) for the determination of bromate in wheat flour has been modified with the object of improving the replicability and accuracy of the results. As in the original method, the extracted bromate is allowed to react with potassium iodide in the presence of starch and the intensity of the color of the resulting starch-iodine solution is measured in a spectrophotometer. With flours containing 5 p.p.m. of bromate the modified method gives average recoveries of 101.5%; with flours containing 10-20 p.p.m., 97%; and with flours containing 35 p.p.m., 95%. The mean difference between duplicate results obtained on different days was 1.14 p.p.m. This difference was influenced very little by the bromate content of the flour, but is believed to depend very largely upon the granulation of the bromate and its distribution.

The procedure described by Hoffer and Alcock (1) for the determination of bromate in flour has not proved generally satisfactory. A critical study of the method made in this laboratory showed that the results were influenced by small differences in technique such as may easily occur between one analyst and another. After trying numerous changes in an effort to secure more consistent results, the following modified procedure was finally adopted.

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Materials and Methods

Apparatus

- (1) 50 ml. centrifuge tubes, without lip.
- (2) Coleman Universal Spectrophotometer, Model 11, and optically matched round cuvettes of 1.7 cm. diameter.

Reagents

- (1) 25% potassium chloride solution.
- (2) Iodine, stock solution. Dissolve 0.35 g. of iodine in 7 ml. of ethyl alcohol and dilute to 1 liter with 1% potassium iodide solution. This solution should be protected from the light.
- (3) Potassium chloride solution, 25% in 2% acetic acid.
- (4) Celite filter aid, analytical grade.
- (5) Standard potassium bromate solution, 5.0 μ g. per ml. Prepare as required from a solution containing 100 μ g. per ml. which is stable.
- (6) 0.5% starch solution, prepared by the following method as outlined by Platner (2): While stirring add approximately 30 ml. of a 20% sodium hydroxide solution to a suspension of 2 g. of soluble starch in 300 ml. of water and allow to stand for 1 hour. Neutralize with concentrated hydrochloric acid using litmus as an indicator and then add 1 ml. of glacial acetic acid. Dilute to 400 ml. with water. This solution can be used for an indefinite period.
- (7) 1% potassium iodide solution.
- (8) Iodine solution, approximately 17 μ g. per ml. Dilute approximately 2.5 ml. of stock iodine solution to 50 ml. Prepare fresh daily.
- (9) 5% sulfuric acid.

Procedure. Weigh 7 g. of flour into a 50 ml. centrifuge tube and add 40 ml. of a mixture consisting of approximately 37.4 volumes of 25% potassium chloride solution and 2.6 volumes of the stock iodine solution. This mixture should be made up just before use. Stopper the centrifuge tube with a rubber stopper and thoroughly disperse the flour by shaking vigorously for 10-15 seconds. Allow to stand for a few minutes and shake again for about 10 seconds. The interval may be utilized for the extraction of other samples in the set. Time of standing is not critical but the shaking must be sufficient to disperse the flour to allow for the solution of the bromate. Centrifuge at 2400 r.p.m. for 10 minutes. Dilute 5 or 10 ml. of the supernatant liquid (or less if the flour contains in excess of 50 p.p.m. of bromate) to 35 ml. with 25% potassium chloride solution in 2% acetic acid and, after

mixing, add from a spatula about 0.8 g. of Celite (as judged by the eye). Stir vigorously with a stirring rod and centrifuge at 2400 r.p.m. for 5 minutes. Pour off all the clear extract and then prepare the following mixtures:

Tube 1 (Reference Solution)	Tube 2	Tube 3
3 ml. distilled water	1 ml. distilled water	1 ml. standard bromate soln.
1 ml. starch soln.	1 ml. starch soln.	1 ml. starch soln.
10 ml. flour extract	10 ml. flour extract	10 ml. flour extract
3 ml. potassium iodide soln.	3 ml. potassium iodide soln.	3 ml. potassium iodide soln.
1 ml. dilute iodine soln.	1 ml. dilute iodine soln.	1 ml. dilute iodine soln.
	2 ml. 5% sulfuric acid	2 ml. 5% sulfuric acid

Each addition is made in rotation, as rapidly as convenient and in the order given. Allow an interval of 2 minutes between the acid additions to tubes 2 and 3, and exactly 4 minutes after adding the acid read the optical density at a wave length of 575 m μ . The difference in optical density between tubes 2 and 3 is due to 5 μ g. of bromate and, when 10 ml. of the first extract is taken, the concentration of bromate in the flour is obtained as follows:

Concentration of bromate,

$$\text{p.p.m.} = \frac{A}{B} \times \frac{35}{10} \times \frac{40}{10} \times \frac{1}{7} \times 5 = \frac{10A}{B}$$

A is the optical density of the unknown in tube (2),

B is the difference in optical density between tubes 3 and 2.

While spectrophotometer readings are being made on one extract, the decanted clear extracts from other samples in the same set can be allowed to stand. They should not, however, be left too long, since slow losses of bromate occur as shown by the following results:

Hours standing	Bromate recovery
0	5.3 p.p.m.
2	5.1 p.p.m.
4	5.0 p.p.m.
6	4.9 p.p.m.

Results and Discussion

The first objective of the efforts to improve the original method was to secure clear extracts as free as possible from flour starch. This was accomplished by extracting with neutral potassium chloride solution, diluting an aliquot with potassium chloride in acetic acid, and removing the precipitate then formed by centrifuging after the addition of Celite. Dilution of the original extract with acid potassium chloride solution is necessary, otherwise precipitation would occur on acidifying at the color development stage. The characteristics of the color produced on adding starch and iodine to clear extracts, obtained in this manner from a variety of flours of different grades and different ages,

were very uniform. With all extracts the maximum light absorption peaks overlapped in the neighborhood of 575 m μ . and any lack of definition of the peaks due to cloudiness was avoided.

Hoffer and Alcock noted that when their method was applied to unbromated flours, optical density readings equivalent to 0.6 p.p.m. of bromate were obtained. The present studies, however, showed the magnitude of this "blank" to be greatly influenced by the time and vigor of shaking. This particular difficulty, which had undoubtedly been responsible for some of the discrepancies in the results of other workers, disappeared on changing the method of extraction. A "blank" was still obtained with unbromated flours but it was small; and in spite of wide variations in the amount of shaking, its value, expressed as μ g. of bromate in the mixture used for color development, only varied from 0.1 to 0.25.

The new extraction method has the further advantage of permitting the use of a larger ratio of flour to extractant, thus reducing the sampling error. Because of the low dosages of bromate used, and the differences in specific gravity and particle characteristics between flour and bromate, it is believed that this error can be quite large.

With the adoption of neutral potassium chloride solution as the bromate extractant, it became necessary to discontinue the use of potassium permanganate since it was no longer reduced. Low bromate recoveries were now obtained. It was then found that on adding increments of a dilute iodine solution to the extract of an unbromated flour, the first addition produced a smaller increase in optical density than subsequent increments. The same thing occurs when iodine is released by increments of bromate; the first increment of bromate gives a low reading. It was, therefore, decided to add a small quantity of iodine to each of the tubes immediately before the addition of acid, and thus ensure that all the bromate from the flour was effective in increasing the color intensity. The exact quantity of iodine added is not important, but it is convenient to use an amount equal to that released by 2 to 2.5 μ g. of bromate.

Upon the introduction of this step bromate recoveries became slightly high. By using extracts of unbromated flours, the high recoveries were found to be due to the fact that the intensity of the starch-iodine color in the tube containing added acid was greater than that in tube 1 containing the reference solution to which no acid is added. As this was not the case when potassium chloride in 2% acetic acid was substituted for flour extract, it was assumed, as a working hypothesis, that soluble substances from the flour took up small quantities of iodine in tube 1, and that this did not occur in the tube containing added acid. In the hope of saturating these soluble substances

with iodine, an excess of iodine was added to the extractant. Whether the hypothesis is correct or not, the use of iodine in the extractant resulted in more satisfactory bromate recoveries. The amount of iodine added is not critical. All that is necessary is to have a visible excess that is adsorbed on the flour and is subsequently removed on centrifuging.

As iodine is slowly liberated when the mixed reagent, used in the original method, is allowed to stand, it was decided to add the acid

TABLE I
RESULTS OF BROMATE DETERMINATIONS ON MIXTURES
OF FLOUR AND BROMATE

Grade of flour	Bromate added, p.p.m.	Bromate found, ¹ p.p.m.	Mean, p.p.m.	Mean recovery, %
Patent	5	4.6	4.7	4.65
Patent	5	4.5	5.6	5.05
Straight	5	5.0	5.5	5.25
Clear	5	4.7	5.8	5.25
Means			5.05	101.0
Patent	12	10.9	11.8	11.35
Patent	12	10.9	11.5	11.2
Straight	12	10.6	13.4	12.0
Clear	12	12.4	12.4	12.4
Means			11.75	97.9
Patent	20	19.0	20.1	19.55
Straight	20	19.0	20.2	19.6
Clear	20	17.7	20.1	18.9
Means			19.35	96.8
Patent	35	34.8	35.0	34.9
Straight	35	34.1	35.9	35.0
Clear	35	29.2	31.2	30.2
Means			33.4	95.3

¹ Duplicate determinations on different days.

and iodide separately. At the same time the concentration of acid was increased in order to speed up the reaction with bromate. After an interval of 3 minutes from the time the stronger acid is added, further increases in optical density take place very slowly. But because they do take place and because there is a very slow fading of the color of the reference solution in tube 1, this short color development time is desirable. Sulfuric acid was substituted for hydrochloric acid because it gave a lower reagent blank. The reagent blank, which should be checked from time to time, should not exceed a value equiva-

lent to 0.25 μ g. of bromate in the mixture of solutions used for color development. If it does, fresh sulfuric acid should be prepared.

The revised method suffers from the same limitation as the original in that it cannot be used when iodates or other substances which release iodine from iodide are present in the flour.

To check the revised method a master mix was prepared by mixing 999 g. of patent flour and 1 g. of potassium bromate in a McLellan mixer for 4 hours. From this, samples of patents, straight and clear,

TABLE II
RECOVERIES OF BROMATE ADDED TO THE EXTRACTANT

Grade of flour	Bromate added, p.p.m.	Bromate found, ¹ p.p.m.	Mean, p.p.m.	Mean recovery, %
Patent	5.7	5.7	5.8	100.9
Patent	5.7	5.5	5.6	97.4
Straight	5.7	5.8	5.8	104.2
Clear	5.7	6.1	6.3	108.8
Means			5.8	102.2
Patent	11.4	10.9	11.1	96.5
Patent	11.4	10.6	10.9	94.3
Straight	11.4	11.0	11.3	97.8
Clear	11.4	11.0	11.3	97.8
Means			11.0	96.6
Patent	34.2	32.4	—	94.7
Patent	34.2	32.1	32.7	94.7
Straight	34.2	32.2	32.6	94.7
Clear	34.2	32.2	32.5	94.6
Means			32.4	94.7

¹ Duplicate determinations on different days.

were made up to contain 5, 12, 20, and 35 p.p.m. of bromate, the mixing of these samples being carried on for 30 minutes. The results are given in Table I.

In a second series, the bromate was added in solution with the potassium chloride-iodine mixture. The results are reported in Table II. The mean difference between the duplicate results was only 0.3 p.p.m. as compared with 1.14 p.p.m. between those given in Table I. Errors involved in diluting the neutral potassium chloride extract, clarifying the diluted solution, and developing the color are very small. Determinations of the bromate present in 10 ml. aliquots of neutral potassium chloride extracts showed that the sum of these errors is responsible for a difference of not more than 0.1 p.p.m. in the results of duplicate bromate determinations on flour. With smaller aliquots

the difference due to these errors would be correspondingly increased because of the larger factor used in the calculation.

From the fact that average recoveries were just as good when bromated flours were analyzed as when the bromate was added with the extractant, it would appear that errors in extraction are not responsible in any substantial measure for the poorer replicability when dealing with bromated flours. The most probable explanation seems to lie in the sampling. After the work reported above had been completed, it was found that the bromate used commercially as a flour improver varies widely in granulation. The material used in this study was of intermediate fineness but the granulation was not uniform. It contained a small fraction of relatively large crystals which failed to pass a 16XX sieve. Some idea of the effect of granulation and the difficulty of dispersing bromate uniformly can be obtained by dropping acidified potassium iodide on smooth flour surfaces and noting the distribution and size of the black spots. A few determinations on flours containing bromate in the form of a very fine powder gave duplicate results which agreed as well as those reported in Table II. This study suggests that particle size will have to be considered in any future work on the bromate determination, for it not only influences the sampling error but is likely to affect recoveries in methods involving sedimentation.

The greater variability in the recovery figures for dry mixed samples is attributed largely to lack of homogeneity of the master mix. These variations are smoothed out in the averages, and average recoveries at the 5, 12, and 35 p.p.m. levels were practically the same in both series. With increasing bromate content, recoveries were lower, averaging 95% at 35 p.p.m.

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EFFECT OF HEREDITY ON THE NIACIN AND PANTOTHENIC ACID CONTENT OF CORN¹

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ABSTRACT

Analyses for niacin and pantothenic acid were made on the inbred lines and single-cross hybrid components of four double-cross hybrids reported in an earlier study. The niacin content of both single- and double-cross hybrids appeared related to that of the inbred lines. It was more difficult to discern a hereditary influence on pantothenic acid, probably because of environmental differences.

Analyses of three inbred lines crossed in various combinations for the study of heterozygosity showed that the two vitamins may behave differently as to inheritance.

Analyses of 36 samples from a group of early, uniform single-cross hybrids, involving nine inbred lines and all grown in approximately the same environmental conditions, showed definite hereditary influence on both niacin and pantothenic acid.

The results in general confirm previous conclusions that the elaboration of both vitamins is influenced by hereditary factors but that the influence on niacin is less subject to modification by environmental factors than is the influence on pantothenic acid. It is suggested that the niacin content of corn can be increased by breeding.

It is recognized that the chemical composition of plants may be affected to a marked extent by both hereditary and environmental factors. Very little is known, however, as to the part which such factors play in the elaboration of vitamins.

Rather wide variations in the amount of several members of the vitamin B complex in corn have been reported by Burkholder *et al.* (2), Teply *et al.* (6), and Ellis and Madsen (4). Recently Aurand and co-workers (1) reported that heredity influenced the carotene content of single-cross corn hybrids. Doty and associates (3) found some indication that the amounts of the various amino acids present in several single-cross hybrids were related to the genetic constitution of the plant.

Previous studies in our laboratory (Hunt *et al.*, 5) showed that both niacin and pantothenic acid in double-cross corn hybrids were influenced significantly by hereditary and environmental factors. The elaboration of pantothenic acid was affected much more by environmental factors than was the elaboration of niacin.

The results in general indicated the need for further investigation of the inbred lines and single-cross hybrids which entered into the

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genetic make-up of the double-cross hybrids. The present report deals with the niacin and pantothenic acid content of various inbred lines and related single- and double-cross hybrids.

Materials and Methods

Analyses were made of the inbred lines and single-cross hybrid components of four double-cross hybrids reported earlier (Hunt *et al.*, 5). These samples were grown during different years at different locations. Another series of samples involved a group of three inbred lines and crosses which was organized for the purpose of studying heterozygosity or plant vigor. The three lines were crossed as single crosses F_1 and F_2 generations, back crosses, three-way crosses, and a fourth type of cross similar to a double-cross hybrid, in which one of the three lines appeared twice (hereinafter designated as "double cross" for the sake of ease of expression). The third series consisted of early, uniform single-cross hybrids obtained by crossing each of nine inbred lines with the other eight lines. The latter two series of corn were grown at the Ohio Agricultural Experiment Station in 1946. Each sample analyzed was a composite of five replications grown in the same field under similar environmental conditions.

The methods of analysis were the same as those used in previous studies (5).

Results and Discussion

The vitamin values for the double-cross hybrids (5) and the component lines and single crosses which enter into their genetic make-up are given in Table I. These samples, as previously stated, were gathered from different seasons and locations, so that some of the variation in vitamin content might be ascribable to environmental factors, especially in the case of pantothenic acid. The four hybrids are related.

There was no significant difference in the average niacin content of the four double-cross hybrids. Among the inbred lines, Oh02 had the highest niacin content (2.91 mg./100 g.), while Ind.WF9, Ind.38-11, and Ill.R4 assayed 2.16, 2.01, and 1.89 mg., respectively. C.I.187-2 (1.56 mg.), Ill.Hy (1.24 mg.), Ia.L317 (1.23 mg.), and Oh40B (1.15 mg.) assayed low in niacin. The single cross highest in niacin, Oh40B \times Oh02, contained the high niacin inbred Oh02. The single cross lowest in niacin (Hy \times L317) contained two low-niacin inbreds. The single crosses composed of the intermediate-niacin inbreds give values between the two extremes.

Slight differences in the niacin content of the double-cross hybrids showed that the hybrid highest in niacin, Ohio C38, contained the high-

niacin inbred, Oh02, one intermediate-niacin inbred (Ind.WF9), and two low-niacin inbreds, Ill.Hy and Oh40B. U.S.35, nearly as high as Ohio C38, contained three intermediate inbreds (Ind.WF9, Ind.38-11, Ill.R4), and one low inbred (Ill.Hy). The hybrids U.S.13 and Ill.201 contained two intermediate and two low inbreds.

TABLE I
NIACIN AND PANTOTHENIC ACID CONTENT OF DOUBLE-CROSS HYBRIDS
AND COMPONENTS (MG./100 G.—10% MOISTURE BASIS)

	Number of samples	Niacin average and P.E.	Pantothenic acid average and P.E.
Ohio C38 (WF9 × Hy) (Oh40B × Oh02)	13	2.13 ± .04	0.54 ± .02
U.S.35 (WF9 × Ind.38-11) (Ill.R4 × Ill.Hy)	14	2.11 ± .03	0.53 ± .02
U.S.13 (Ill.Hy × Ia.L317) (Ind.WF9 × Ind.38-11)	14	1.95 ± .04	0.49 ± .03
Ill.201 (Ind.WF9 × Ind.38-11) (C.I.187-2 × Ia.L317)	14	2.02 ± .03	0.56 ± .03
Oh02	3	2.91 ± .07	0.61 ± .01
Oh40B	3	1.15 ± .04	0.53 ± .04
Ind.WF9	4	2.16 ± .08	0.74 ± .03
Ind.38-11	5	2.01 ± .09	0.77 ± .06
Ill.R4	1	1.89	0.45
Ill.Hy	6	1.24 ± .04	0.49 ± .07
Ia.L317	4	1.23 ± .03	0.52 ± .04
C.I.187-2	3	1.56 ± .04	0.59 ± .04
Oh40B × Oh02	1	2.92	0.52
Ind.WF9 × Ill.Hy	1	2.12	0.19
Ind.WF9 × Ind.38-11	1	2.01	0.50
Ill.R4 × Ill.Hy	1	2.09	0.53
Ill.Hy × Ia.L317	1	1.38	0.39
C.I.187-2 × Ia.L317	1	1.48	0.53

It appears from these data that the niacin content of both the single-cross and double-cross hybrids is related to the niacin content of their component inbred lines.

The average pantothenic acid assay values for the double-cross hybrids were similar. Inbreds Ind.WF9 and Ind.38-11 averaged

higher in pantothenic acid than the other inbred lines, with C.I.187-2 next in line. The double-cross hybrid highest in pantothenic acid, III.201, has these three inbreds in its makeup. However, the pantothenic acid content of the double-cross hybrids did not resemble as closely the pantothenic acid content of their components as was the case with niacin. Previous work (Hunt *et al.*, 5) has shown that pantothenic acid is influenced markedly by environmental factors which may mask some of the effects of hereditary factors.

TABLE II
NIACIN AND PANTOTHENIC ACID CONTENT OF SAMPLES FROM STUDY OF
HETEROZYGOSITY (MG./100 G.—10% MOISTURE BASIS)

Type of sample		Niacin	Pantothenic acid
Inbred lines	51A ¹	2.20	0.59
	56A ¹	1.74	0.62
	40B ¹	.95	0.60
Single cross hybrids <i>F</i> ₁ generation	51A × 56A	2.10	0.66
	51A × 40B	1.78	0.57
	40B × 56A	1.97	0.73
Same, <i>F</i> ₂ generation	51A × 56A	2.01	0.76
	51A × 40B	1.74	0.63
	40B × 56A	1.65	0.68
Back crosses	(51A × 56A) (51A)	2.15	0.66
	(51A × 56A) (56A)	1.95	0.77
	(51A × 40B) (51A)	2.01	0.56
	(51A × 40B) (40B)	1.34	0.60
	(40B × 56A) (40B)	1.49	0.75
	(40B × 56A) (56A)	1.85	0.85
Three-way crosses	(51A × 56A) (40B)	2.06	0.63
	(51A × 40B) (56A)	2.15	0.69
	(40B × 56A) (51A)	2.26	0.59
Double crosses	(51A × 56A) (40B × 56A)	1.88	0.88
	(51A × 40B) (51A × 56A)	1.98	0.52
	(51A × 40B) (40B × 56A)	1.73	0.63

¹ 51A, 56A, and 40B are all Ohio hybrids.

The results of the analyses of the group of lines and various types of crosses organized for the study of heterozygosity are shown in Table II. The three inbred lines Oh51A, Oh56A, and Oh40B differed markedly in niacin content, while the variations in this vitamin are not so pronounced in the hybrids. The single crosses, both *F*₁ and *F*₂ generations, the three-way crosses, and the double crosses all had niacin values which appeared more closely related to Oh51A and Oh56A than to Oh40B. The single crosses *F*₁ generation had slightly higher niacin than the *F*₂ generation. The back crosses with Oh51A

and Oh56A also were similar to these lines in niacin content, whereas those with Oh40B showed a lowering of niacin, though neither was as low as the inbred itself. With the exception of the three-way cross, in which Oh51A was one parent, the niacin values of the hybrids all fell between the limits established by Oh51A and Oh40B, the highest and lowest inbreds, respectively.

TABLE III

NIACIN AND PANTOTHENIC ACID CONTENT OF SINGLE CROSS CORN HYBRIDS
(MG./100 G.—10% MOISTURE BASIS)

Inbred			III.A			A153			A158		
Crossed with	Niacin	Panto. acid									
III.A	1.94	0.54	III.A	1.94	0.54	III.A	2.21	0.41	A153	2.22	0.40
A153	2.21	0.41	A158	2.22	0.40	A153	2.22	0.40	WR3	2.66	0.22
WR3	2.16	0.50	WR3	1.90	0.54	WR3	2.66	0.22	W3	2.66	0.27
W3	2.56	0.38	W3	2.47	0.32	W3	2.66	0.27	W22	2.22	0.23
W22	2.20	0.46	W22	2.21	0.32	W22	2.22	0.23	Oh51A	2.61	0.25
Oh51A	2.29	0.45	Oh51A	2.03	0.23	Oh51A	2.61	0.25	B8	2.59	0.31
B8	2.39	0.41	B8	2.49	0.31	B8	2.59	0.31	B9	1.89	0.34
B9	1.13	0.43	B9	1.62	0.29	B9	1.89	0.34	Mean	2.38	0.30
Mean	2.11	0.45	Mean	2.11	0.37	Mean	2.38	0.30			
Inbred			WR3			W3			W22		
III.A	2.16	0.50	III.A	2.56	0.38	III.A	2.20	0.46	A153	2.21	0.32
A153	1.90	0.54	A153	2.47	0.32	A153	2.22	0.23	A158	2.22	0.23
A158	2.66	0.22	A158	2.66	0.27	A158	2.22	0.23	WR3	2.38	0.34
W3	2.09	0.37	WR3	2.09	0.37	WR3	2.38	0.34	W3	3.04	0.29
W22	2.38	0.34	W22	3.04	0.29	W22	3.04	0.29	Oh51A	2.46	0.30
Oh51A	2.10	0.41	Oh51A	2.70	0.32	Oh51A	2.46	0.30	B8	3.11	0.28
B8	2.58	0.32	B8	3.04	0.42	B8	3.11	0.28	B9	1.99	0.40
B9	1.74	0.36	B9	1.94	0.31	B9	1.99	0.40	Mean	2.45	0.33
Mean	2.20	0.38	Mean	2.56	0.34	Mean	2.45	0.33			
Inbred			Oh51A			B8			B9		
III.A	2.29	0.45	III.A	2.39	0.41	III.A	1.13	0.43	A153	1.62	0.29
A153	2.03	0.23	A153	2.49	0.31	A153	1.62	0.29	A158	1.89	0.34
A158	2.61	0.25	A158	2.59	0.31	A158	1.89	0.34	WR3	1.74	0.36
WR3	2.10	0.41	WR3	2.58	0.32	WR3	1.74	0.36	W3	1.94	0.31
W3	2.70	0.32	W3	3.04	0.42	W3	1.94	0.31	W22	1.99	0.40
W22	2.46	0.30	W22	3.11	0.28	W22	1.99	0.40	Oh51A	2.02	0.32
B8	2.74	0.41	Oh51A	2.74	0.41	Oh51A	2.02	0.32	B8	2.13	0.40
B9	2.02	0.32	B9	2.13	0.40	B9	2.13	0.40	Mean	1.81	0.36
Mean	2.37	0.34	Mean	2.63	0.36	Mean	1.81	0.36			

The pantothenic acid content of the three inbred lines was nearly the same. The range of pantothenic acid values for the whole study was not as wide as the range of niacin values. Several hybrids yielded higher pantothenic acid than did the inbred lines, and variations were

more pronounced among the hybrids than among the lines. In these respects pantothenic acid differed from niacin. The line Oh56A seemed responsible for highest pantothenic acid, Oh40B intermediate, and Oh51A for lowest pantothenic acid in back crosses, three-way crosses, and double crosses. The highest pantothenic acid value obtained was in the double-cross hybrid in which Oh56A appeared twice; the lowest value was in the double-cross hybrid in which Oh51A appeared twice.

Table III shows the niacin and pantothenic acid content of the early, uniform single-cross hybrids. These data are arranged so that each inbred appears as a common parent crossed with eight other inbred lines. The data were analyzed statistically according to the following procedure: Group comparisons were made for each inbred line with each other inbred line by omitting, in each two groups, the single-cross hybrid containing the two lines which were being compared. For example, in the two groups which compared the effect of lines III.A and A153, the single cross III.A \times A153 was omitted. The results of the statistical analysis are shown in Tables IV and V.

TABLE IV

EFFECT OF INBRED LINES ON NIACIN CONTENT OF SINGLE CROSS HYBRIDS

- Line B8 higher than W3, W22, A158, Oh51A, WR3*, III.A*, A153**, B9**
- Line W3 higher than W22, A158, Oh51A, WR3, III.A*, A153*, B9**
- Line W22 higher than A158, Oh51A, WR3, III.A, A153, B9**
- Line A158 higher than Oh51A, WR3, III.A, A153, B9**
- Line Oh51A higher than WR3, III.A, A153, B9**
- Line WR3 higher than III.A, A153, B9**
- Line III.A higher than B9** (same as A153)
- Line A153 higher than B9**
- Line B9 lower than all other lines

* Significant.

** Highly significant.

TABLE V

EFFECT OF INBRED LINES ON PANTOTHENIC ACID CONTENT OF SINGLE CROSS HYBRIDS

- Line III.A higher than WR3, A153*, B8**, B9**, Oh51A**, W3**, W22**, A158**
- Line WR3 higher than A153, B8, B9, Oh51A, W3, W22, A158*
- Line A153 higher than B8, B9, Oh51A, W3, W22, A158
- Line B8 higher than Oh51A, W3, W22, A158 (same as B9)
- Line B9 higher than Oh51A, W3, W22, A158
- Line Oh51A higher than W22, A158 (same as W3)
- Line W3 higher than W22, A158
- Line W22 higher than A158
- Line A158 lower than all other lines

* Significant.

** Highly significant.

The line B8 as the common parent produced single-cross hybrids having the highest niacin content. This effect was significant when compared with III.A and WR3 and highly significant when compared

with B9 and A153. Hybrids containing B9 assayed lowest in niacin; the difference between B9 and each other line was highly significant. The single cross B8 \times W22 contained the highest niacin (3.11 mg./100 g.) and B9 \times Ill.A contained the lowest (1.13 mg.).

The line Ill.A as the common parent produced single-cross hybrids having the highest pantothenic acid content. This effect was significant when compared with A153 and highly significant when compared with B8, B9, 51A, W3, W22, and A158. Hybrids containing A158 assayed the lowest in pantothenic acid. The difference between A158 and the other lines was significant with WR3 and highly significant with Ill.A. The single crosses A153 \times Ill.A and WR3 \times A153 contained 0.54 mg. per 100 g., the highest pantothenic acid value; the lowest value, 0.22 mg. per 100 g., was found in the single cross WR3 \times A158.

In the analysis of uniform single-cross hybrids, both niacin and pantothenic acid were influenced definitely by hereditary factors.

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EFFECT OF BENTONITE ON LOAF VOLUME AND WEIGHT OF HARD AND SOFT WHEAT BREAD¹

NETTIE C. ESELBAUGH²

ABSTRACT

The responses of a soft and a hard wheat flour to naturally occurring bentonite (Volclay, B. C. Dust) as it affects loaf volume and weight were investigated. Both the straight dough and sponge and dough processes of making bread were used.

The loaf volume of breads made from a hard wheat flour progressively increased as the amounts of bentonite were increased up to 0.8 or 1.0%. These breads compared favorably in color and texture with bread containing 2 mg. % potassium bromate. On the other hand, loaf volume decreased with increasing amounts of bentonite when a soft whole wheat flour was used in the straight dough process.

The observed increase in loaf volume is not due to any action of the bentonite on the yeast. A bentonite-treated yeast suspension produced a loaf which was smaller than the control.

The weight of the loaf tended to show a slight increase as the content of bentonite increased. This is no doubt due to a slightly higher water content.

Different theories, supported by experimental observation, have been advanced in an effort to understand the beneficial effects of certain oxidizing compounds on the texture and loaf volume of bread. The mechanism by which these flour improvers may act has been explained principally in two different ways, viz., the retardation of proteinase activity inherent in the flour and/or their action on certain reducing groups or bonds in the flour proteins or other dough ingredients. Hildebrand (5) has very ably reviewed the numerous reports in the literature on this subject.

Earlier work by the present writer (4) indicated that the proteolytic activity of papain was retarded in the presence of bentonite (Volclay, B. C. Dust). Free titratable acidity in a gluten-papain suspension was increased 13 times above the initial value during an incubation period of four days compared to an increase of only seven times in the gluten-papain-bentonite suspension. Corresponding increases in the amino acid content (8) were six times for the gluten-papain suspension, four times for the gluten-papain-bentonite suspension. Could this inorganic material act as a proteinase inhibitor and hence a flour improver? The observations of Ensminger and Giesecking (2) de-

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scribing the adsorption of proteins by montmorillonitic clays appeared further to justify raising this question.

Observations on the effect of bentonite on the loaf volume and weight of both a hard and a soft wheat bread are reported. In addition the total reduced nitrogen and total solids of the water extract, as well as the swelling capacity of the bread crumb made from a hard whole wheat flour, are given.

Materials and Methods

Breads with varying amounts of bentonite were prepared by both the straight dough and the 50% sponge and dough processes. Recognizing that differences in either the protein content or the proteolytic activity of flours, as well as the method of manipulation of the doughs, might influence any responses to the bentonite treatment, both soft wheat and hard wheat flours were used.

Basic ingredients were:

	<i>Straight dough process</i>	<i>Sponge and dough process</i>	
	<i>Sponge</i>	<i>Dough</i>	
Flour	200	100	100
Nonfat milk solids	8	8	—
Shortening (hydrogenated vegetable)	6	—	6
Sodium chloride	3	—	3
Yeast (compressed)	6	6	—
Sucrose	12	8	4
Water—amount for optimum absorption	—	—	75 ml.
Bentonite—variable per cent based on the weight of the flour	—	—	—
Potassium bromate—2 mg. % based on the weight of the flour (in only those mixes as indicated in the tables)	—	—	—

Manipulation of Doughs:

1. Straight dough process: The dough was punched every 5 minutes for 20 minutes, given a 15-minute rest period, panned, proofed for another 65 minutes, and baked.
2. Fifty per cent sponge and dough process: The sponge was fermented 3.5 hours at $36.5^{\circ}\text{C.} \pm 1.5^{\circ}$, mixed with the dough ingredients, fermented for another 50 minutes, punched, given a 15-minute rest period, panned, proofed for 50 minutes, and baked.

Doughs made from the above ingredients were mixed by a mechanical mixer, divided into halves, proofed at $36.5^{\circ}\text{C.} \pm 1.5^{\circ}$ in a constant temperature cabinet, and baked in a reel type oven at 232°C. (450°F.) for 23 minutes. One to two hours after removing from the oven, weight and volume of the separate loaves were observed.

Tests on Bread Crumb:

1. Swelling capacity was determined by the technique of Cathcart and Luber (1) with slight modification. Tests were made on the 2-hour-old bread.
2. Total water-soluble nitrogen—determined by the micro-Kjeldahl method on the water extract obtained from the swelling capacity test.
3. Total solids of water-extract—aliquots of the centrifugate from the determination of the swelling capacity were air-dried at 100°C. \pm 1° for 6 hours, cooled, and weighed.

Results and Discussion

With increasing increments of bentonite up to 0.8% of the flour used, loaf volume progressively increased with a hard wheat flour (Table I). Even with 0.2% added bentonite a volume greater than

TABLE I

EFFECT OF BENTONITE ON LOAF VOLUME AND WEIGHT OF A HARD WHEAT BREAD¹

Bentonite (%)	KBrO ₃ (mg. %)	Straight dough process ²		Sponge and dough process	
		Weight (g.)	Volume (ml.)	Weight (g.)	Volume (ml.)
—	—	159.0	532	160.8	462
0.2	—	164.2	542	162.8	492
0.4	—	163.5	617	158.5	520
0.6	—	157.2	632	160.5	538
0.8	—	170.0	710	163.2	545
1.0	—	—	—	167.0	540
—	2.0	159.2	655	155.8	495

¹ Average values of duplicate loaves.

² Bran removed with 40-mesh sieve.

that for the control was obtained. Loaves were superior in volume to that of the bromated breads (2 mg. % potassium bromate) with 0.6 to 0.8% bentonite using the straight dough process; with 0.2 to 0.4% bentonite using the sponge and dough process. Loaf weight also increased. Furthermore, the crumb color, aroma, and texture of the breads with 0.6 to 1.0% bentonite compared favorably with the bromated breads (Fig. 1). With either method of fermentation the treated breads had thinner cell walls, lighter crumb, and lighter color than the controls (A-2 and B-1) which contained neither bentonite nor bromate. The volumes of the bentonite-treated bread (A-10 and B-11) were even greater than those of the bromated loaves (A-11 and B-13).

The effect of bentonite on the soft whole wheat flour was opposite to that observed with the hard wheat flour: a decrease in loaf volume

with the former, an increase in the latter (Table II). Cell walls of the crumb of the bread made from the soft whole wheat flour were thick, heavy, and of a coarse texture.

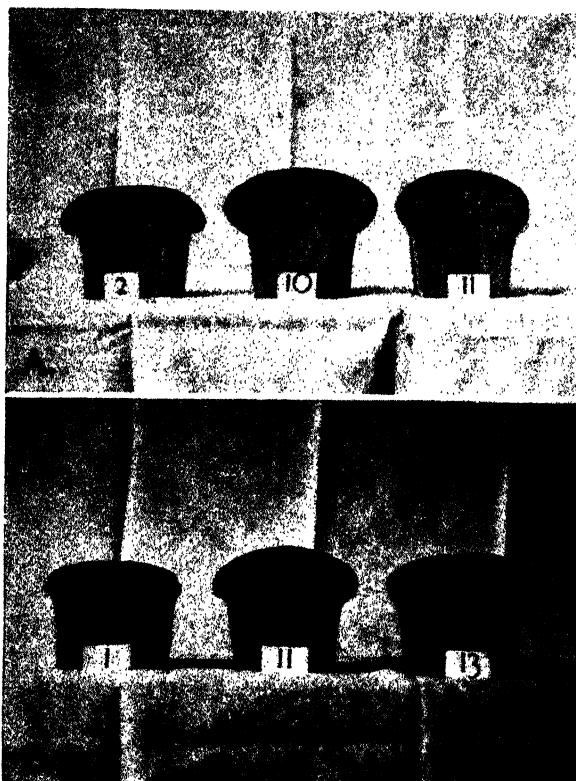


Fig. 1. Effect of bentonite on loaf volume and texture using a hard wheat flour.

- A. Straight dough process
 - Loaf 2—Control, no bentonite or bromate
 - Loaf 10—0.8% bentonite
 - Loaf 11—2 mg. % bromate
- B. 50% sponge and dough
 - Loaf 1—Control, no bentonite or bromate
 - Loaf 11—1.0% bentonite
 - Loaf 13—2 mg. % bromate

Evidence points toward retarded protein degradation in the bentonite-treated bread (Table III). In freshly baked breads made from a hard whole wheat flour, there was less reduced nitrogen in the water extract of the bentonite-treated breads than in either the bromated or control breads. Likewise, the total solids in the water extract of the bentonite-treated breads were less than either the bromated or control breads.

On the other hand, the swelling capacity of the bentonite-treated bread was higher than that of the others. Doughs to which increasing amounts of bentonite were added required increasing amounts of water to secure an optimum absorption of moisture. No doubt the large water-absorbing capacity of montmorillonitic clays explains not only the increased swelling capacity but also the increased weight of the loaves containing bentonite.

TABLE II
EFFECT OF BENTONITE ON LOAF VOLUME AND WEIGHT
AS INFLUENCED BY QUALITY OF FLOUR PROTEIN

Bentonite (%)	Straight dough process ¹			
	Soft whole wheat flour		Hard wheat flour ²	
	Weight (g.)	Volume (ml.)	Weight (g.)	Volume (ml.)
—	144.0	348	159.0	532
0.2	146.0	322	164.2	542
0.4	147.0	350	163.5	618
0.6	148.0	280	157.2	632
0.8	138.0	262	170.0	710

¹ Average values of duplicate loaves.

² Bran removed with 40-mesh sieve.

TABLE III
TOTAL NITROGEN AND SOLIDS OF THE WATER EXTRACT AND THE
SWELLING CAPACITY OF BREAD CRUMB MADE FROM A
HARD WHOLE WHEAT FLOUR¹

Treatment	Water-soluble nitrogen (mg. %)	Total solids of water extract ² (g.)	Swelling capacity ² (ml.)
Control	225.7	8.8	286.7
KBrO ₃	220.5	8.8	238.0
2 mg. % Bentonite			
0.4%	206.5	7.8	292.2
0.8%	181.8	7.6	346.3

¹ Sponge and dough process.

² Per 100 g. bread crumb.

However, these observations do not explain the difference in the response of the soft wheat and hard wheat flours to the added bentonite.

To determine whether the favorable action of bentonite was on the yeast or the flour, bread leavened with bentonite-treated yeast was made from hard wheat flour. The yeast and 0.4% bentonite were intimately mixed with an electric mixer.

Following refrigeration at 8°C. \pm 2° for 10 hours, the bentonite-yeast suspension was used in making bread by the sponge and dough

process. Yeast for the control and bromated breads was treated in the same manner except that no bentonite was added to the suspension. The loaf volumes were as follows: control loaves, 452 cc.; bromated loaves, 505 cc.; bentonite loaves, 392 cc. That the smaller loaf volume is due to the bentonite treatment of the yeast and not to injury of the yeast cells by the severe mechanical treatment is shown by the normal volumes of the control and bromated breads. Some constituent necessary for the metabolism of yeast may have been irreversibly adsorbed onto the clay during the 10 hours standing in the refrigerator. The work of Ensminger and Giesecking (3) indicates that as a protein molecule is adsorbed in an acid medium as a cation, the base exchange capacity of the bentonite is definitely reduced. In this way the clay may either have inactivated the yeast cells or have been rendered inactive itself in respect to its beneficial effects.

In the slightly acid medium in which fermentation of the bread doughs occurs, it is probable that "powerful but latent" proteinases (6), or some of the flour proteins, are positively charged. As such, and acting as cations, they might be adsorbed onto the negatively charged bentonite. If the labile radical of a proteinase was thus removed from the field of activity, its proteolytic action on the flour proteins would be retarded.

If similar reasoning is applied to the flour proteins they should be less subject to proteolytic attack or their stability increased toward reducing agents inherent in fermenting dough. The work of Olcott, Sapirstein, and Blish (7) would appear to support this last theory. On the other hand, the beneficial effect may result from the adsorption of reducing groups formed during the mixing and fermentation.

However, the foregoing assumptions fail to account for the response of the soft whole wheat flour to bentonite. It is possible that these observations can be explained by a greater degree of solvation of the flour proteins, due to the large water-binding capacity of bentonite. The extent of solvation might have produced an optimum elasticity in the hard wheat flour but was too great for the soft wheat flour. This would result in a weakened flour protein and a smaller loaf of bread for the soft wheat flour.

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A NOTE ON THE EFFECT OF CHLORINE-SUBSTITUTED PHENOXY COMPOUNDS ON THE MILLING AND BAKING QUALITY OF HARD RED SPRING WHEAT¹

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ABSTRACT

The work described shows that application of the acetate, triethyl amine, and butyl ester of 2,4-dichlorophenoxyacetic acid to wheat plants at different stages of growth did not impair milling and baking quality. There was some evidence that the protein content and baking quality were improved by some of the treatments.

Organic substances possessing growth-promoting activity are becoming increasingly important in the field of plant research (2, 3). With the discovery of the selective action of the different compounds emphasis has become concentrated on weed-killing action. Since 1934 more than one thousand derivatives of phenoxy acetic acid and similar substances have been tested as growth regulators or weed killers.

The purpose of this preliminary study was to ascertain if any harmful effects upon milling and baking quality would result from the use of 2,4-dichlorophenoxy compounds on growing wheat. Five varieties of hard red spring wheat were included in the present inquiry. Applications of three 2,4-dichlorophenoxy compounds were made when the plants were at the tiller stage in one experiment. In a second experiment the compounds were added at four different stages of plant growth of one wheat variety only. The wheat was planted on weed-free, hand-cultivated plots, consisting of three 18-foot rows 12 inches apart, and replicated four times. Sixteen feet of the center row of each plot was used for this study. The compounds were added in aqueous solution adjusted to yield one pound of acid equivalent per acre. The wheat harvested from these plots was milled and baked by micro methods (1) and the wheat protein content ascertained (Kjeldahl-Gunning method).

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The data and tentative conclusions secured from this study are presented in this note.

Table I shows the mean results obtained from the five wheat varieties from the plots treated with the three phenoxy compounds noted. These compounds appeared to affect all the five varieties in

TABLE I
EFFECT OF THREE PHENOXY COMPOUNDS ON MILLING AND BAKING QUALITY

Treatment ¹	Protein content	Flour yield	Loaf volume	Crumb color
	%	%	cc.	
A	14.9	68.8	172	7.1
B	14.8	69.1	173	7.1
C	15.5	68.1	183	7.1
D	15.5	68.3	191	7.2

¹ A = No treatment.

B = Sodium 2,4-dichlorophenoxy acetate.

C = 2,4-dichlorophenoxytriethylamine.

D = Butyl ester of 2,4-dichlorophenoxyacetic acid.

the same manner regarding milling and baking quality; therefore the original data are not shown.

Treatments with 2,4-dichlorophenoxytriethylamine and the butyl ester of 2,4-dichlorophenoxyacetic acid increased the protein content approximately 0.6%; the loaf volume was also slightly increased. The other two criteria of quality were not affected by the treatments. The sodium salt had no effect.

The results from the treatment at different growth stages are shown in Table II. These data are means of results obtained by applications

TABLE II
EFFECT OF STAGE OF APPLICATION OF THREE PHENOXY COMPOUNDS
ON MILLING AND BAKING QUALITY

Stage	Protein content	Flour yield	Loaf volume	Crumb color
	%	%	cc.	
Control	15.1	71.3	175	8.0
Tiller	15.7	71.1	188	8.5
Boot	15.9	70.2	148	7.8
Bloom	16.2	70.8	168	8.0
Soft dough	15.3	71.3	157	8.0

of the three compounds listed in Table I with one wheat variety (Mida). Application at the tiller stage increased protein content and loaf volume in a comparable manner with the results shown in Table II; however, in this instance loaf crumb color was improved. Treatment at the bloom stage increased protein content most, but had no effect on loaf volume. Application at the boot stage produced 0.8% more protein than the control, but yielded the lowest loaf volume.

Application of the phenoxy compounds to wheat during the various growth stages had no effect on the mixogram pattern.

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1. SIBBITT, L. D., SCOTT, G. M., and HARRIS, R. H. A further comparison of flours obtained with the micro and Allis-Chalmers mills. *Cereal Chem.* **20**: 679-685 (1943).
2. THIMANN, K. V. Currents in biochemical research. 21. Plant hormones and the analysis of growth. *Interscience: New York* (1946).
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BOOK REVIEW

On the Structure of the Protein Molecule. A Chemical Investigation. By N. Trönsgaard. Second edition. 124 pp. Einar Munkgaard, Copenhagen, Denmark, publisher; G. E. Stechert and Co., 31-37 East Tenth Street, New York City. 1944. Price \$4.50.

The author has for some years been convinced that at least the globular proteins possess properties which are not consistent with a structure of long polypeptide chains. He points out that the customary hydrolysis to the constituent amino acids is a drastic treatment; consequently he resorts to a mild reduction with sodium in amyl alcohol as a means of stabilizing the linkages before a mild hydrolysis to obtain the protein fragments. He has identified isoamyl amine and several heterocyclic rings containing nitrogen among the products obtained; certain other fractions have been isolated and analyzed in the form of their platinic chloride salts. Among the proteins studied are wheat gliadin and the albumin, globulin, and globin fractions of horse serum.

This monograph is a summary of his findings over a period of some twenty years. From these he is led to postulate that the most characteristic feature of the structure of proteins consists of heterocyclic subunits of essentially the same elementary composition; these units are linked together by short aliphatic chains. His picture has something in common with the Wrinch cyclol theory and with Abderhalden's diketopiperazine postulate. Trönsgaard's conclusions are not the currently accepted views; nevertheless he has done considerable careful analytical work which must be considered in formulating any theory of protein structure. For this reason the book is recommended to anyone concerned with this still unsettled problem.

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SUGGESTIONS TO AUTHORS

General. From January 1, 1948, an abstract will be printed at the beginning of each paper instead of a summary at the end, references will be numbered to provide the option of citing by number only, and date of receipt, author's connections, etc., will be shown in footnotes. Except on these points, authors will find the last volume of *Cereal Chemistry* a useful guide to acceptable arrangements and styling of papers. "On Writing Scientific Papers for Cereal Chemistry" (*Trans. Am. Assoc. Cereal Chem.* 6: 1-22, 1948) amplifies the following notes.

Authors should submit two copies of the manuscript, typed double spaced with wide margins on 8½ by 11 inch white paper, and all original drawings or photographs for figures. If possible, one set of photographs of figures should also be submitted. Originals can then be held to prevent damage, and the photographs can be sent to reviewers.

Titles and Footnotes. Titles should be specific, but should be kept short by deleting unnecessary words. The title footnote shows "Manuscript received . . ." and the name and address of the author's institution. Author footnotes, showing position and connections, are desirable although not obligatory.

Abstract. A concise abstract of about 200 words follows title and authors. It should state the principal results and conclusions, and should contain, largely by inference, adequate information on the scope and design of the investigation.

Literature. In general, only recent papers need be listed, and these can often be cited more advantageously throughout the text than in the introduction. Long introductory reviews should be avoided, especially when a recent review in another paper or in a monograph can be cited instead.

References are arranged and numbered in alphabetical order of authors' names and show author, title, journal, volume, first and last pages, and year. The list is given at the end of the paper. Reference numbers must invariably be cited in the text, but authors' names and year may be cited also. Abbreviations for the names of journals follow the list given in *Chemical Abstracts* 40: I-CCIX, 1946.

Organization. The standard organization involves main sections for abstract, introduction, materials, methods, results, discussion, acknowledgments, and literature cited. Alternately, a group of related studies, each made with different materials or methods, may require a separate section for each study, with subsections for materials and methods, and for results, under each section. Center headings are used for main sections and italicized run-in headings for subsections, and headings should be restricted to these two types only.

Tables. Data should be arranged to facilitate the comparisons readers must make. Tables should be kept small by breaking up large ones if this is feasible. Only about eight columns of tabular matter can be printed across the page. Authors should omit all unessential data such as laboratory numbers, columns of data that show no significant variation, and any data not discussed in the text. A text reference can frequently be substituted for columns containing only a few data. The number of significant figures should be minimized. Box and side headings should be kept short by abbreviating freely; unorthodox abbreviations may be explained in footnotes, but unnecessary footnotes should be avoided. Leader tables without a number, main heading, or ruled lines are often useful for small groups of data.

Tables should be typed on separate pages at the end of the manuscript, and their position should be indicated to the printer by typing "(TABLE I)" in the appropriate place between lines of the text. (Figures are treated in the same way.)

Figures. If possible, all line drawings should be made by a competent draftsman. Traditional layouts should be followed: the horizontal axis should be used for

the independent variable; curves should be drawn heaviest, axes or frame intermediate, and the grid lines lightest; and experimental points should be shown. Labels are preferable to legends. Authors should avoid identification in cut-lines to be printed below the figure, especially if symbols are used that cannot readily be set in type.

All drawings should be made about two to three times eventual reduced size with India ink on white paper, tracing linen, or *blue*-lined graph paper; with any other color, the unsightly mass of small grid lines is reproduced in the cut. Lettering should be done with a guide using India ink; and letters should be $\frac{1}{8}$ to $\frac{1}{4}$ inch high after reduction.

For difficult photographs, a professional should be hired or aid obtained from a good amateur. The subject should be lighted to show details. A bright print with considerable contrast reproduces best, and all prints should be made on glossy paper.

All original figures should be submitted with one set of photographic reproductions for reviewers, and each item should be identified by lightly writing number, author, and title on the back. Cut-lines (legends) should be typed on a separate sheet at the end of the manuscript. "Preparation of Illustrations and Tables" (*Trans. Am. Assoc. Cereal Chem.* 3: 69-104. 1945) amplifies these notes.

Text. Clarity and conciseness are the prime essentials of a good scientific style. Proper grouping of related information and thoughts within paragraphs, selection of logical sequences for paragraphs and for sentences within paragraphs, and a skillful use of headings and topic sentences are the greatest aids to clarity. Clear phrasing is simplified by writing short sentences, using direct statements and active verbs, and preferring the concrete to the abstract, the specific to the general, and the definite to the vague. Trite circumlocutions and useless modifiers are the main causes of verbosity; they should be removed by repeated editing of drafts.

Editorial Style. A.A.C.C. publications are edited in accordance with *A Manual of Style*, University of Chicago Press, and *Webster's Dictionary*. A few points which authors often treat wrongly are listed below:

Use names, not formulas, for text references to chemical compounds. Use plural verbs with quantities (6.9 g. were). Figures are used before unit abbreviations (3 ml.), and % rather than "per cent" is used following figures. All units are abbreviated and followed by periods, except units of time, which are spelled out. Repeat the degree sign (5°-10° C.). Place 0 before the decimal point for correlation coefficients ($r = 0.95$). Use * to mark statistics that exceed the 5% level and ** for those that exceed the 1% level; footnotes explaining this convention are no longer required. Type fractions on one line if possible, e.g., $A/(B + C)$. Use lower case for farinograph, mixogram, etc., unless used with a proper name, i.e., Brabender Farinograph. When in doubt about a point that occurs frequently, consult the Style Manual or the Dictionary.

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LOAF VOLUME AND PROTEIN CONTENT OF HARD WINTER AND SPRING WHEATS¹

KARL F. FINNEY and MARK A. BARMORE²

ABSTRACT

Loaf volume data for samples covering a wide range in protein content are reported for hard winter and hard spring wheat varieties and for commercial samples representing four crops and grown under a wide range of climatic and soil conditions. A highly bromated, rich formula containing milk solids was employed in conjunction with optimum mixing time and water requirement.

The major factor accounting for variation in loaf volume within a variety was protein content, and the relation between these two factors was essentially linear between the limits of protein encountered, i.e., 8 to 18%. Certain varieties, however, had distinctly different regression lines, the slopes of which, in general, increased as the loaf volume level became greater. The increase in loaf volume for each percentage increase in protein varied from about 40 cc. to 75 cc. for different varieties.

The loaf volume-protein content regression lines for varieties represent differences in protein quality. The loaf volume level for different varieties at 13.5% protein varied from 823 cc. to 1015 cc., a difference several times that required for statistical significance. Using the variety regression lines, a logical method of correcting loaf volumes for differences in protein content has been developed and used successfully for evaluating new and old varieties of hard winter and hard spring wheats.

Regression lines for loaf volume on protein content clearly demonstrate that the best quality hard winter and hard spring wheat varieties are equal in protein quality or breadmaking capacity as are also the medium and poor quality varieties of the two classes.

One of the principal tasks of the Hard Winter Wheat Quality Laboratory is to characterize experimental and commercial varieties of hard

¹ Manuscript received April 21, 1948.

The studies herein reported are a part of the cooperative work carried on by the Division of Cereal Crops and Diseases, Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture, and the agricultural experiment stations of the Great Plains Region, the Department of Milling Industry, Kansas Agricultural Experiment Station, and the Department of Agronomy, Ohio Agricultural Experiment Station.

² Chemists, Bureau of Plant Industry, Soils, and Agricultural Engineering, formerly stationed at the Hard Winter Wheat Quality Laboratory, Kansas Agricultural Experiment Station, Manhattan, Kansas, and the Federal Soft Wheat Laboratory, Ohio Agricultural Experiment Station, Wooster, Ohio; now at the State Agricultural Experiment Stations at Manhattan, Kansas, and Pullman, Washington, respectively.

red winter wheat so that they may be evaluated for use in the mill and bakeshop. The commercial suitability of new varieties is based on the similarity of their properties to those of the well-known commercial varieties. Because both hard winter and hard spring wheats are used primarily for bread flours, quality research on either class should be supplemented by a comparison with the other. Accordingly, representative hard spring wheat varieties have been included in the hard winter wheat research program since 1940.

One of the important properties that determines the suitability of a new variety of wheat for making bread is the relation of loaf volume to protein content. This paper presents the results of a four year study on the relation between protein content and loaf volume of bread for each of 14 hard winter and 8 hard spring wheat varieties. The relative loaf volume potentialities of hard red winter and hard red spring wheat flours are discussed together with the application of the data to the evaluation of new and experimental varieties of wheat. Subnormal loaf volumes, not accounted for by variety or protein content, are identified and classified according to flour mixing requirement.

Review of Literature

Early work failed to show a linear relation between protein content and loaf volume. The data of Thomas (21), Shollenberger (20), and others indicated that with increasing protein content there was a diminishing increase in loaf volume up to 13 to 15% protein, depending on the class of wheat. Beyond 13 to 15% there was no increase in some instances and a sharp decline in others. Bailey and Sherwood (2), from data obtained on the 1921 to 1925 crops, inclusive, calculated the formula of the curve representing the relation between loaf volume and protein content. They found it to be hyperbolic, thus indicating that "each increment of increase in protein content results in a diminished increment of increase in loaf volume."

Thus, low correlation coefficients, many of which, however, were significant, were obtained previous to the work of Larmour (12) who obtained a correlation coefficient of 0.906 for loaf volume and protein content of Canadian hard red spring wheat grown in one season. Using composite samples representing the normal protein range, he concluded that the curvilinearity of the relation between protein content and loaf volume was limited to the extreme protein levels, and that the regression of loaf volume on protein was linear between the limits of 7% and 15.9%. Aitken and Geddes (1), using composite samples of Canadian spring wheat flour, found that the relation of loaf volume to protein content was approximately linear over the entire range of 11.4% to 15.7%.

Although most of the early work was carried out with comparatively lean baking formulas and small amounts of bromate, several recent publications describe baking methods employing a rich, highly bromated formula and approaching optimum conditions. All except Larmour (14) failed, however, to include optimum oxidation at the same time nonfat milk solids and shortening were present in the formula. Some did not use one or more of the following: shortening, nonfat milk solids, malt, or optimum mixing time. Finney and Barmore³ (9) emphasized the importance of the combination of certain amounts of these ingredients and also showed (10) that the lack of proper mixing leads to erroneous conclusions for some varieties. The formula described by them and used with minor changes by a large number of workers gives loaf volumes which are an accurate expression of the quality and quantity of protein in the flour. With this type of formula, a linear relation between loaf volume and protein content has been reported by several workers.

Larmour, Working, and Ofelt (15), using composite samples of Turkey, Kanred, and Blackhull, representing one season, obtained a correlation between loaf volume and protein content of +0.98. Sandstedt and Ofelt (19) made a study of the comparative quality of certain wheat varieties at various naturally occurring protein levels representing one season. They found that the loaf volume and protein content were approximately linear within a variety. Similarly, the results of Bayfield, Working, and Harris (4), Bayfield and West (3), and Johnson, Swanson, and Bayfield (11) have shown that for a given season loaf volume and protein content were linear within a variety. Although correlation coefficients were not given, they were obviously high.

Finney (7), by means of a fractionating and reconstituting technique whereby the factors of soil and climate were eliminated, studied the relation between loaf volume and protein content over the range of about 0% to 20% protein, using varieties of wheat which differed in quality of protein. This work showed that loaf volume and protein content were linear between the limits of 7% or 8% to at least 20% protein. Below 7% protein, however, the relation was definitely curvilinear, all curves meeting at 0% protein and about 275 cc. loaf volume. The regression of loaf volume on protein content was different for different varieties and appeared to be a function of the loaf volume that was produced by a variety at any arbitrary protein level within the range of linearity. Regression lines for loaf volume on protein content suggested a fan-shaped family of lines that could be applied to determine the factor for correcting the loaf volume for a given sample to a constant protein basis from a knowledge of its protein content and loaf volume.

³ A study of baking methods for evaluating the protein quality of hard winter wheats. Presented at annual AACC meeting, 1939.

Larmour (13) summarized the published information on hard spring and winter wheats and concluded that there was no evidence of fundamental difference in baking quality. Additional evidence in support of this view was obtained by Larmour (14).

Materials and Methods

As part of the wheat improvement program carried out by the U.S. Department of Agriculture in cooperation with the state agricultural experiment stations, commercial and new varieties are grown throughout the hard winter and spring wheat regions for study. A complete description of the field studies is given by Quisenberry (17) and Clark (5). Grain of these varieties from carefully studied and widely different environments representing the hard wheat region was made available for milling and baking investigations. Thirty-one stations in 13 states covering the central United States from Texas to the Dakotas have supplied grain during at least one of the four years 1940-43, inclusive. Many have not been able to supply grain each year owing to poor conditions of one sort or another.

Nine samples of Thatcher grown in Saskatchewan and Alberta, Canada, in 1941 and 1942 were also included. A composite sample of commercially milled Thatcher flour was supplied each year by the Northwest Crop Improvement Association from its test plots in the spring wheat region.

The older varieties such as Nebred, Minturki, Tenmarq, Kharkof, Blackhull, Cheyenne, Yogo, Early Blackhull, Chiefkan, Pilot, Thatcher, Rival, and Marquis have all been described by Clark and Bayles (6). The newer varieties, Comanche and Pawnee, have been described by Reitz and Laude (18). Several of the newer varieties listed have been described previously only in mimeographed reports. Of these, Wichita has been recommended as a commercial variety in Oklahoma, Texas, and Kansas; Red Chief is a new wheat produced by Earl Clark, a private breeder of Sedgwick, Kansas; Merit and Premier are two new spring wheats that were included in the earlier studies but probably will not be released for commercial production.

For the study of the relation of milling and baking characteristics to market grade of hard winter wheat, the Federal Grain supervisors of the Grain Branch, Production and Marketing Administration, U. S. Department of Agriculture, furnished composite samples of the major market grades collected at the principal terminal markets in the winter wheat region during each of three crop years.

All samples except Thatcher from the Northwest Crop Improvement Association were obtained as wheat and milled experimentally on a Buhler or Allis mill. The unbleached flours were stored at about 70°F.

for 3 weeks and then at about 40°F. for one or more months until baked. The baking method involved the use of optimum mixing time, and the following rich, highly bromated formula: flour 100 g., water as needed, sugar 6 g., salt 1.5 g., shortening 3 g., yeast 2 g. (standardized), nonfat milk solids 4 g., 120°L. malt syrup 0.25 g., and potassium bromate 1 to 5 mg. The loaf volume for a variety was that for the optimum amount of bromate. The doughs were fermented for 3 hours and proofed for 55 minutes at 86°F. The baking method is described in more detail by Finney and Barmore (9 and 10).

Differences in loaf volume of 20 to 25 cc. are required for significance when using the same lot of yeast. When using a given lot of flour, but using different shipments of yeast, 30 to 35 cc. differences are required for significance, even when the largest variations in yeast are eliminated according to the procedure of Finney and Barmore (8). Another error of about 25 to 35 cc. may be introduced due to the effect of slight variations in experimental milling on loaf volume. Thus it would be possible for these different errors to be all positive or negative on any one sample, thereby accounting for occasional loaf volume fluctuations about a varietal regression line of as much as 70 cc.

Results

The relation between loaf volume and protein content for the hard winter varieties is shown graphically in Figs. 1 and 2 and for the hard springs in Fig. 3. A letter has been used to identify the source of each sample. The lines shown are the regression lines, the equations for which are given in Table I along with other pertinent statistical data.

Hard Winter Wheat Varieties. Certain hard winter samples tested during the four year period reported here were regarded as subnormal in mixing time, dough-handling properties, and loaf volume and unsatisfactory for quality evaluation, just as are samples with extremely low test weights. Since the subnormal loaf volumes are definitely accounted for by factors other than variety and protein content, statistical calculations were made both including and excluding these subnormal samples. Subnormal samples are identified in Figs. 1 and 2 and the data and discussion pertaining to them are presented later. The correlation coefficients given in Figs. 1 and 2 were obtained when the subnormal samples were omitted.

The relation between loaf volume and protein content is essentially linear within a variety (Figs. 1 and 2). Considering the large number of individual samples representing several crop years, and the extremes in environment for each variety, the correlation coefficients are more highly significant than those obtained by previous investigators. These data show that protein content accounts for nearly 90% of the varia-

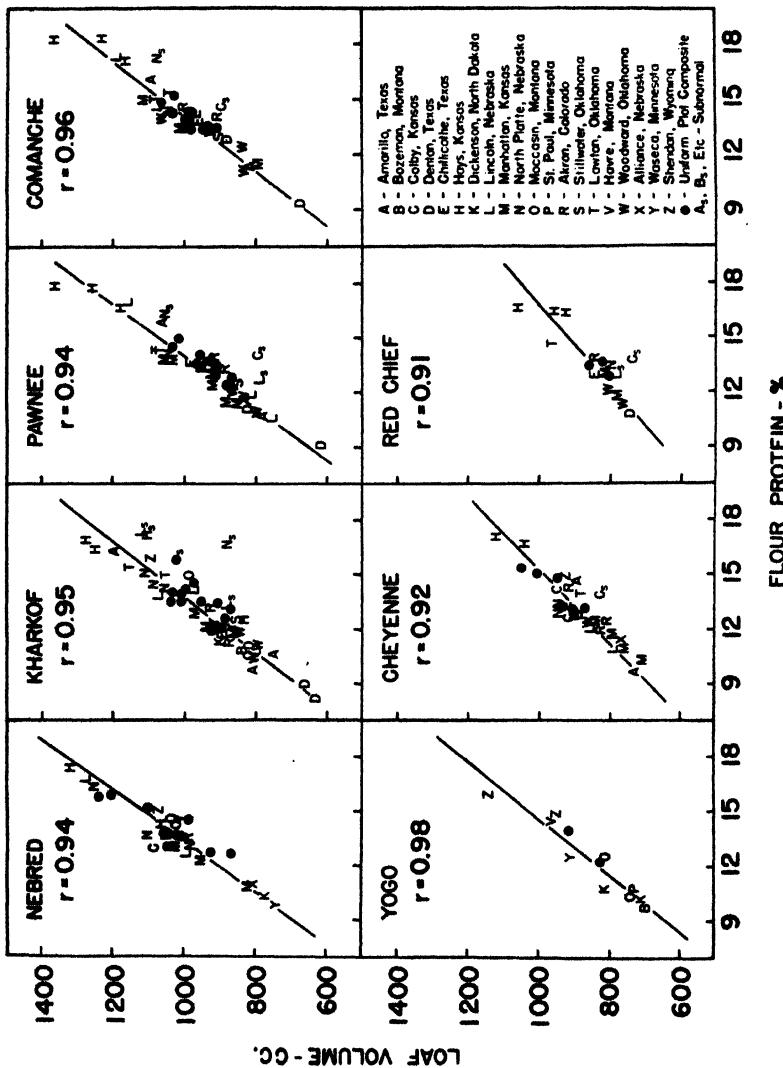


Fig. 1. Loaf volume and protein content for seven hard winter wheat varieties grown for several seasons under widely different conditions of climate and soil. Key identifies each sample as to source.

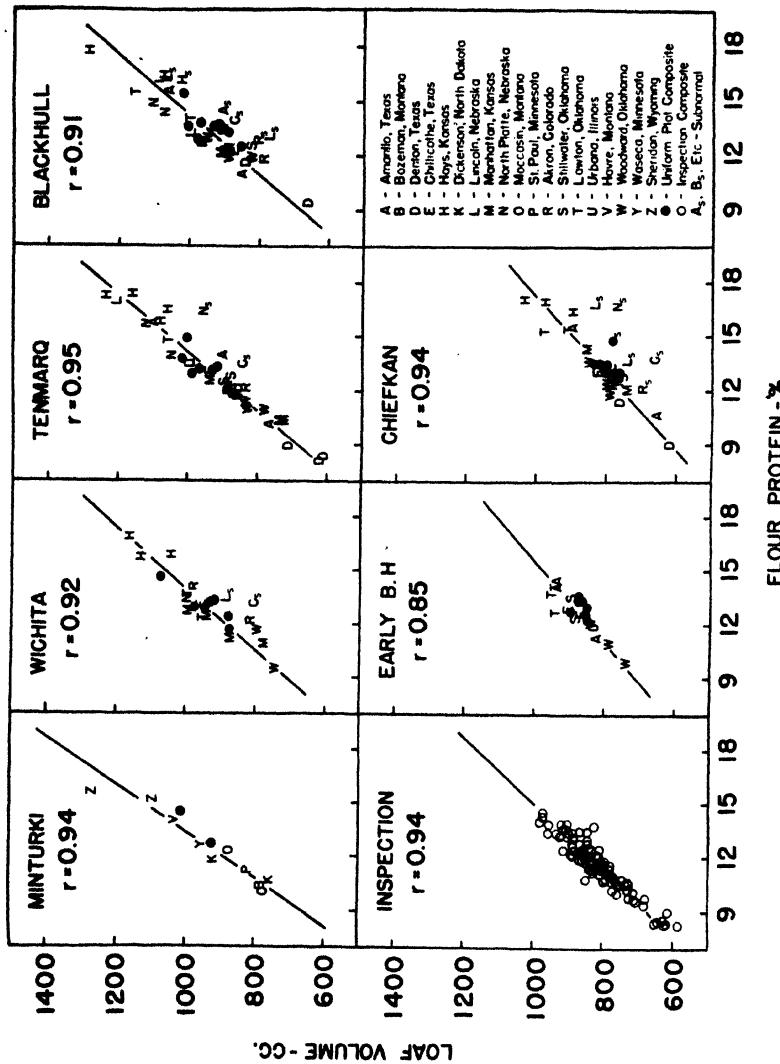


Fig. 2. Loaf volume and protein content for inspection samples and six hard winter wheat varieties grown for several seasons under widely different conditions of climate and soil. Key identifies each sample as to source.

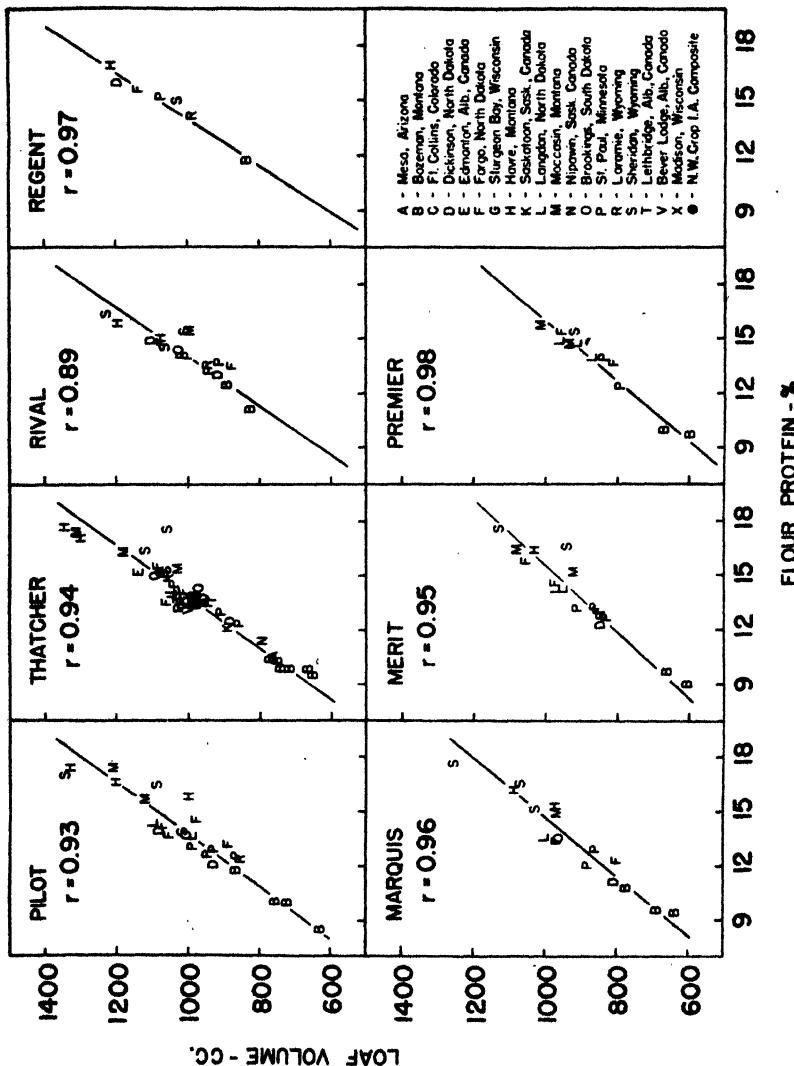


Fig. 3. Loaf volume and protein content for seven hard spring wheat varieties grown for several seasons under widely different conditions of climate and soil. Key identifies each sample as to source.

TABLE I
STATISTICAL SUMMARY OF PROTEIN CONTENT AND LOAF VOLUME DATA FOR HARD WINTER AND HARD SPRING WHEAT VARIETIES

Variety	C.I. number	Crop years (incl.)	No. samples	Corr. coeff. (<i>r</i>)		Regression equation ¹	Mean flour protein ²	Mean loaf volume ³	Loaf volume ⁴	
				Without subnormal samples	All samples				cc.	cc.
Hard Winter Varieties										
Nebred	10094	1940-42	30	0.94	0.94	73.15X + 30.6	13.6	1029	1018	1201
Minturki	6155	1940-42	12	0.95	0.95	75.19X - 8.4	12.5	935	1007	1211
Kharukof	1442	1940-43	47	0.95	0.89	65.33X + 102.6	12.8	938	985	1148
Wichita	11952	1940-43	22	0.92	0.87	58.86X + 177.7	13.0	943	972	1120
Pawnee	11669	1940-43	39	0.95	0.92	70.94X + 8.9	13.3	953	967	1142
Tenmarq	6936	1940-43	38	0.95	0.92	61.76X + 129.2	12.9	925	963	1117
Comanche	11673	1940-43	36	0.96	0.95	67.13X + 55.0	14.0	997	961	1129
Blackhull	6251	1940-43	33	0.91	0.87	61.23X + 127.2	13.5	954	954	1107
Yogo	8033	1940-42	12	0.95	0.95	62.36X + 88.5	12.3	854	930	1086
Inspection	—	1940-42	114	0.94	0.94	54.41X + 181.9	11.6	814	917	1053
Cheyenne	8885	1940-43	30	0.92	0.90	49.78X + 243.6	13.1	894	916	1040
E. Blackhull	8856	1940-43	21	0.85	0.85	43.76X + 317.3	12.8	875	908	1018
Red Chief	12109	1942-43	15	0.91	0.85	43.90X + 261.1	13.5	854	854	964
Chiefkan	11754	1940-43	27	0.94	0.76	46.94X + 189.6	13.4	819	823	941
Hard Spring Varieties										
Pilot	11945	1940-43	—	—	0.93	68.99X + 55.3	13.7	1002	987	1159
Thatcher	10003	1940-43	—	—	0.94	70.10X + 30.5	13.6	989	977	1152
Rival	11708	1941-43	—	—	0.89	74.05X - 42.3	14.2	1008	958	1143
Regent	12070	1943	—	—	0.97	78.56X - 107.9	15.0	1068	953	1149
Marquis	3641	1940-42	—	—	0.96	61.24X + 105.6	13.4	925	932	1085
Merit	11870	1940-42	—	—	0.95	54.41X + 155.4	13.9	914	890	1026
Premier	11940	1940-41	—	—	0.98	60.30X + 32.4	13.7	859	846	997
Progress	6902	1940-41	—	—	0.99	40.50X + 293.5	13.5	838	840	942

¹ Loaf volume vs. flour protein (X) without subnormal samples (all hard spring data used).

² Omitting samples with critically subnormal mixing times (all hard spring data used).

³ Calculated from regression equation.

tions in loaf volume within a variety irrespective of the season and such environmental factors as soil and climate, if the samples identified by critically subnormal mixing times are excluded.

The differences between the regression lines (subnormal samples omitted) for the hard winter wheat varieties are strikingly illustrated in Fig. 4 in which the regression lines for all varieties are shown. The re-

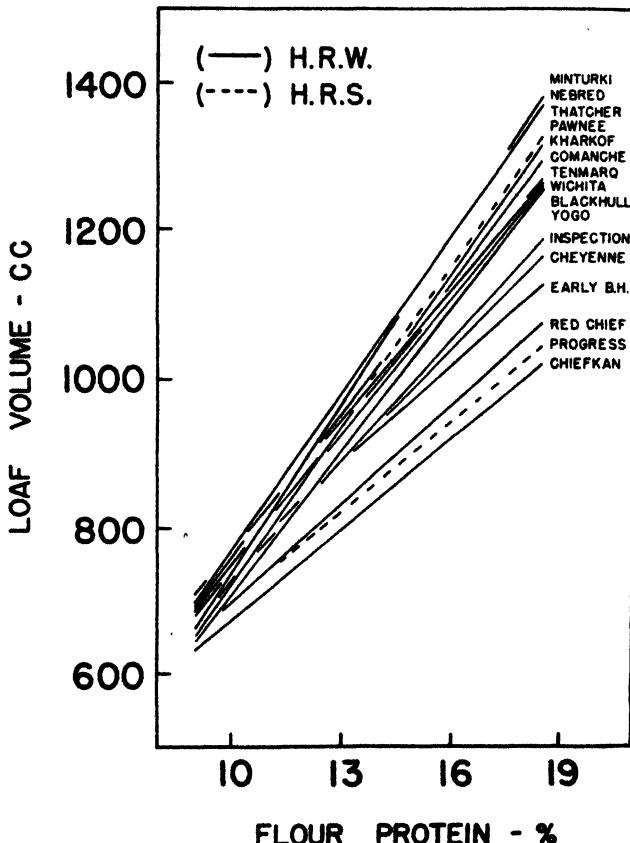


Fig. 4. Loaf volume-protein content regression lines for 13 hard winter wheat varieties, the inspection composites, and two hard spring wheat varieties.

gression lines for two varieties of spring wheat, Thatcher and Progress, are included for comparison. The data show that, in general, the regression coefficients or slopes of the regression lines increase as loaf volume level increases. Thus the increase in loaf volume for each per cent increase in protein varies from 46.9 cc. for Chiefkan, which has the smallest loaf volume, to 73.2 and 75.2 cc. for Nebred and Minturki, respectively, the two varieties that gave the largest loaf volumes. This "fanning-out" of the regression lines is shown to be real

by the very highly significant correlation coefficient $r = 0.94$, obtained when their slopes were compared with the corresponding loaf volumes at 16% protein. When the slopes for the regression lines for all samples were correlated with the corresponding loaf volumes at 16% protein, an equally significant value $r = 0.95$, was obtained.

By analysis of covariance the slopes of the variety regression lines for all samples were found to be heterogeneous ($P \ll 0.001$). This fact justified the testing for significance of various pairs of slopes within the group of 14 regression lines, and it was found that the slope of Chiefkan differed significantly from those for Nebred, Pawnee, Comanche, and the Inspection samples ($P \leq 0.05$). Other pairs of slopes that differed statistically include Early Blackhull with Nebred and Minturki; Cheyenne with Nebred; and Inspection with Minturki, Nebred, and Pawnee ($P \leq 0.05$). Several other pairs of slopes approached statistical significance. When the subnormal samples were omitted in a similar analysis, the slopes for the group of regression lines likewise were found to be heterogeneous ($P \ll 0.001$). The slopes of Chiefkan, Early Blackhull, and Inspection differed significantly from the slopes of Minturki, Nebred, Pawnee, and Comanche. Likewise, the slopes for Cheyenne and Nebred and for Early Blackhull and Kharkof differed significantly ($P \leq 0.05$). In addition, the differences between the slopes for numerous other pairs of varieties approached significance.

The loaf volumes for certain varieties are considerably different, especially at the high protein levels. At 13.5% protein, for example, the loaf volume for Chiefkan is 823 cc. and for Nebred 1018 cc., a difference of nearly 200 cc. At 16% protein, however, the difference between Nebred and Chiefkan is about 260 cc. Thus greater differentiation is obtained at the higher protein levels. Each of the more important loaf volume differences at 16% protein was compared with the corresponding standard error of the difference. Since the sampling variation of the regression coefficients was ignored, larger values for "t" at the 5%, 1%, and 0.1% level of significance were used than would have been otherwise necessary. For example, a value for "t" ordinarily regarded as significant at the 1% point was interpreted as indicating significance at the 5% point. The difference of 59 cc. between Nebred and Pawnee at 16% protein was significant at the 1% point but was interpreted as being significant only at the 5% point. In general a loaf volume difference of 50 to 60 cc. between two varieties was interpreted as being significant at the 5% point; whereas a difference of about 100 cc. was significant even beyond the 0.1% level. The loaf volume difference of 260 cc. between Nebred and Chiefkan was 13.9 times the standard error of the difference and accordingly was significant far beyond the 0.1% level. Out of 64 loaf volume differences at 16% protein that were

statistically significant ($P \leq 0.05$), 44 were significant at $P \leq 0.001$. Even when the subnormal samples were included, 56 loaf volume differences between various pairs of varieties were statistically significant ($P \leq 0.05$), 33 of which were significant at $P \leq 0.001$.

The ranking of this group of hard winter wheat varieties by their regression lines corresponds with their generally recognized loaf volume potentialities. Accordingly, loaf volumes on a constant protein basis can be used as a reliable index of relative breadmaking capacity or protein quality. For a comparison of loaf volumes not on a comparable protein basis, these variety regression lines can be used to correct loaf volume to a constant protein basis. About 13.5% protein is the average of all samples encountered and thus should be the most satisfactory constant basis for keeping the amount of correction to a minimum.

Loaf volume on a constant protein basis (which is regarded as a measure of protein quality), however, is by no means the only property which determines the suitability of a variety for bread-baking purposes. Although probably the most important, it must be considered in conjunction with other important properties such as wheat hardness and milling quality, dough mixing, oxidation, and water requirements, and bread crumb grain and color scores.

Hard Spring Wheat Varieties. The relation between loaf volume and protein content for the spring wheat flours, presented in Fig. 3, is also essentially linear within a variety. The correlation coefficients show that protein content accounts for fully 90% of the variations in loaf volume within a variety, or slightly more than for hard winter wheat regardless of the season, soil, and climate.

The differences between the regression lines for loaf volume on protein content for the hard spring wheat varieties are illustrated in Fig. 5, which also includes regression lines for two hard winter varieties for comparison. As was true for the hard winter wheats, the slopes of the regression lines, in general, increase as loaf volume increases. Referring to Table I, the increase in loaf volume for each per cent increase in protein varies from 40 cc. for Progress, which has the smallest loaf volume, to 74 cc. for Rival and 78 cc. for Regent, two varieties possessing superior loaf volume potentialities. Regent, however, is represented by only one crop year. This "fanning-out" of the regression lines is shown to be real by the very highly significant $r = 0.92$, obtained when their slopes were compared with the corresponding loaf volumes at 16% protein.

Analysis of covariance indicated that the slopes for the various hard red spring regression lines were not heterogeneous. Hence it appears that the "fanning-out" of the regression lines is not sufficiently pronounced to be statistically significant in terms of a test for nonhomogeneity.

geneity of the sampling slopes. The hard spring wheat samples generally were smaller in number than those for the hard winter wheats. That fact probably has contributed to the failure to find statistically significant heterogeneity of the slopes of the regression lines in spite of a very highly significant correlation of 0.92 between slope and loaf volume at 16% protein.

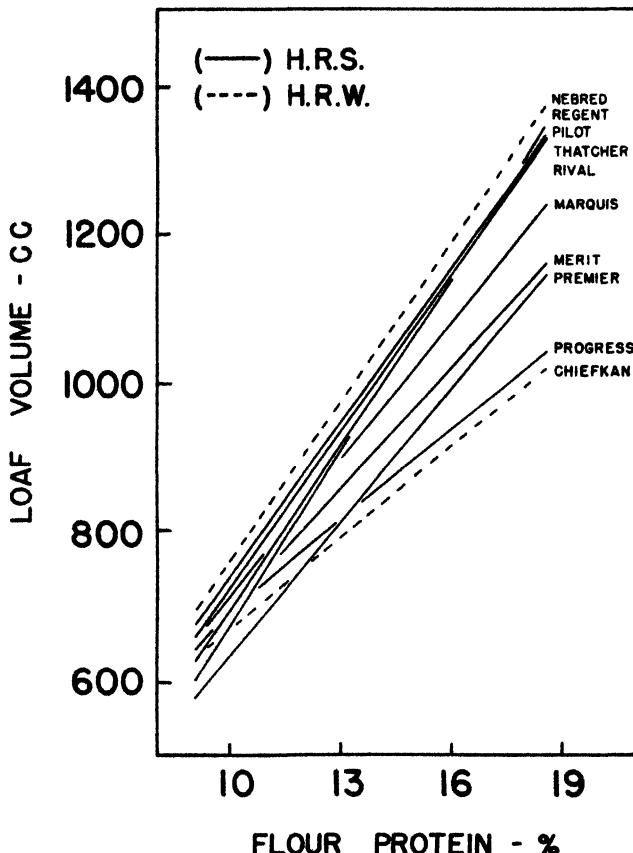


Fig. 5. Loaf volume-protein content regression lines for eight hard spring and two hard winter wheat varieties.

Large loaf volume differences exist between certain hard spring varieties at the higher protein levels. For example, at 16% protein the loaf volume for Progress is only about 940 cc. in contrast to about 1150 cc. for Pilot, Thatcher, Regent, and Rival. Each of the more important loaf volume differences at 16% protein was compared with the corresponding standard error of the difference. In general, a loaf volume difference of 40 to 50 cc. between two varieties was significant at the 5% point; whereas a difference of about 90 cc. was significant at or be-

yond the 0.1% level. The loaf volume difference of 162 cc, between Thatcher and Premier was 9.7 times the standard error of the difference for these two varieties and accordingly was significant far beyond the 0.1% level. Out of 21 loaf volume differences at 16% protein that were statistically significant ($P \leq 0.05$), 16 were significant at $P \leq 0.001$.

Discussion

Application of Regression Lines for Loaf Volume on Protein Content. The relatively small number of samples of some varieties, particularly of the hard red spring class, and the high variances of estimate of others make it difficult to establish some differences in slope that probably are real. Nevertheless, the heterogeneity of the slopes for the hard red winters taken as a whole, the very high correlation coefficients for slope and loaf volume at 16% protein for both winters and springs, the significance of some and the near significance of other differences between the slopes of various pairs of varieties, and the very highly significant differences in loaf volume levels with high protein content leave little room for doubt that loaf volume differs for different varieties and for different protein levels to a degree and in such a manner as to warrant the application of these data to experimental baking and the evaluation of new and experimental varieties of wheat.

Protein content of hard wheats usually varies from about 8 to 20% depending on the climate and soil. Varieties grown presumably under identical conditions have differed by as much as 2% in protein content, which might account for as much as 150 cc. difference in loaf volume. In order to measure protein quality it is necessary that these variations in protein content be taken into account. The obvious way to do this is to adjust loaf volumes to a constant protein level by means of the regression lines. It is of fundamental importance to note that if the regression lines of Figs. 4 and 5 are extended they tend to pass through a common origin within the limits of experimental error at about 7% protein. A necessary consequence of this is that the regression lines of all varieties represent a family of lines of varying slopes which increase with loaf volume at any given protein level. By making use of this fact, it is possible to construct a correction chart showing the regression lines for all possible varieties regardless of protein quality. Such a graph is shown in Fig. 6.

Obviously, the value of the application of Fig. 6 lies in the ability to compare the relative protein quality of new and of established varieties on a constant protein basis. Since loaf volume for a given protein content is a characteristic of the variety and determines its regression line, the loaf volume and protein content of a flour of unknown variety can be used to identify that variety as regards its general protein-quality

class. For example, flour from an unknown variety has a protein content of 15% and gives a loaf volume of 900 cc. The point on Fig. 6 determined by this protein content and loaf volume is located on the regression line whose slope is 45. Following down this line to 13.5% protein gives a loaf volume of about 835 cc. which, according to Fig. 4, is midway between the loaf volumes of Red Chief and Chiefkan at 13.5% protein. The unknown flour is now described as having poor pro-

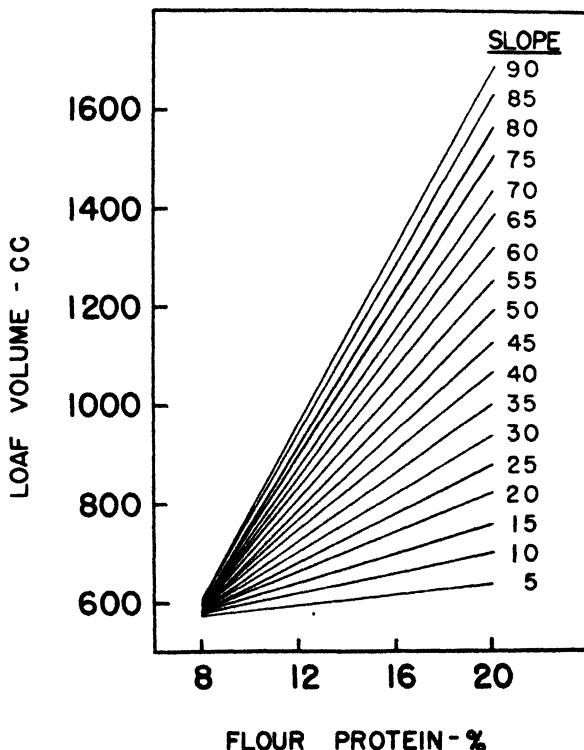


Fig. 6. Chart for correcting loaf volume to a constant protein basis.

tein quality about equal to that of Red Chief or Chiefkan. The protein level of 13.5% used for comparison in the above example is about the average of all samples tested. When correcting the loaf volumes of any particular group of samples, however, it is desirable to use their mean protein content as the constant protein basis, and thereby apply a minimum of correction to the group as a whole.

The application of this procedure of correcting loaf volumes for differences in protein content is not without error and especially so when correcting the loaf volumes of extremely low protein samples to a relatively high protein basis such as 13.5%. Nevertheless, it is derived

logically from the data at hand and is more accurate than a correction factor based on an average of the regression lines of many varieties.

This method of correcting loaf volumes should not be applied to results obtained by baking methods which are not optimum or are lacking in some important ingredient. It might apply, however, to other formulas if it can be shown that the location and slopes of the regression lines are similar to those shown in Figs. 4, 5, or 6. For example, the regression lines reported by Larmour (14) fit very well, as do those of Johnson, Swanson, and Bayfield (11) when using a similar formula. The results of Larmour (14) and McCalla (16) obtained by means of the malt-phosphate-bromate formula, however, are not in agreement. It is quite possible that the goodness of fit of other regression lines to the general family of lines in Fig. 6 may be a measure of whether or not the baking method used was optimum.

Comparison of Hard Winter and Hard Spring Wheat Varieties. This study of hard winter and hard spring wheat varieties for several seasons makes possible an accurate comparison of the protein quality of the two classes of wheat. The regression lines for loaf volume on protein content of two hard spring wheat varieties have been included with those for the hard winters of Fig. 4; and those for two winters have been included with the regression lines for the hard springs of Fig. 5. An examination of those figures and the loaf volume data at 13.5% protein given in Table I shows that the loaf volume potentialities of representative hard spring wheat varieties covers about the same range as that of hard winter wheats. For example, Thatcher and Pilot appear to be very nearly equal to Nebred and Minturki and slightly better than Pawnee and Comanche; Marquis is equal to Blackhull and slightly below Kharkof, Wichita, and Tenmarq; Merit is similar to Cheyenne, whereas Premier is somewhat poorer than Early Blackhull; and Progress is about midway between the two poorest hard winter wheats, Red Chief and Chiefkan. Larmour (13 and 14) came to this same general conclusion after summarizing the published information on hard winter and spring wheats and conducting baking tests on one crop of both classes of wheat. In determining the suitability of a wheat variety for bread-making purposes, as has been previously pointed out, the property of loaf volume-producing ability at a given protein level (regarded as an index of protein quality) must be considered in conjunction with other important properties such as wheat hardness and milling quality; dough mixing, oxidation, and water requirements; and bread crumb grain and color scores.

Subnormal Samples. Occasionally the flours milled from wheats grown at certain stations have been characterized by subnormal mixing requirements. When the degree of subnormality reaches a certain

critical point, two significant facts have been noted in connection with such samples, namely, the dough-handling properties are comparatively poor and the loaf volumes are always considerably below normal.

Recent studies to be presented in a later publication indicate that these subnormal mixing times and loaf volumes are a function of high temperatures during the fruiting period. Thus, in studying the relation

TABLE II

LOAF VOLUME FOR VARIETY SAMPLES CHARACTERIZED BY SUBNORMAL
MIXING TIMES, MANY OF WHICH ARE CRITICALLY SUBNORMAL

Variety	Mixing time		Loaf volume		Mixing time		Loaf volume	
	As rec.	Expected ¹	As rec.	Expected ²	As rec.	Expected ¹	As rec.	Expected ²
	min.	min.	cc.	cc.	min.	min.	cc.	cc.
NORTH PLATTE—1940					AKRON—1942			
Kharkof	1 1/8	2 3/4	881 ³	1160	1 5/8	2 1/4	928	950
Blackhull	1 3/8	2	1072	1123	1 1/2	2	805	910
Tenmarq	1 1/8	3 1/4	958	1145	2 3/4	3 1/4	888	875
Cheyenne	—	— ⁴	—	—	3 1/4	4 3/8	845	845
Nebred	2 1/4	3 1/8	1263	1230	2 3/4	3 1/8	1003	1025
Pawnee	1 1/2	2 1/8	1055	1165	1 3/4	2 1/8	895	945
Comanche	1 1/2	3 1/8	1075	1210	2 1/8	3 1/8	1010	1028
Chiefkan	1 1/8	2 1/8	768	942	1 1/2	2 1/8	698	758
Wichita	—	— ⁴	—	—	2	2 3/4	978	1000
COLBY—1943					LINCOLN—1943			
Kharkof	—	— ⁴	— ⁴	—	1 3/8	2 1/4	885	955
Blackhull	1 1/4	2	868	970	1 1/4	2	765	915
Tenmarq	2	3 1/4	843	957	2 1/2	3 1/4	888	903
Cheyenne	3	4 3/8	827	935	3 1/4	4 3/8	852	845
Pawnee	1 5/8	2 1/8	795	950	1 1/2	2 1/8	790	880
Comanche	1 5/8	3 1/8	893	1035	1 7/8	3 1/8	960	995
Chiefkan	1 1/4	2 1/8	658	800	1 1/8	2 1/8	738	790
Wichita	1 3/8	2 3/4	805	950	1 1/8	2 3/4	882	965
Chey. x Tq.	1 5/8	3 3/8	788	900	2 1/4	3 3/8	885	865
Red Chief	1 1/2	2 3/8	735	855	1 3/8	2 3/8	778	825

¹ Average of several years data.

² Expected on basis of protein content of samples and variety regression line.

³ Definitely subnormal loaf volumes are italicized.

⁴ Not available for testing.

of loaf volume to protein content within and between varieties, it appears imperative that the effect of high temperature during the fruiting period on mixing time and loaf volume be considered. This has been accomplished to a limited degree in the work reported here by considering separately all wheat samples which are characterized by critically subnormal mixing times.

Practically all samples that have been characterized as having seriously subnormal mixing times during the crop years reported in this paper were grown at North Platte, Nebraska, in 1940, Colby, Kansas, in 1943, and Lincoln, Nebraska, in 1943. Samples representing an appreciably less degree of mixing time subnormality than those just mentioned were grown at Akron, Colorado, in 1942. The mixing times and loaf volumes, both as received and expected, for the samples from these four stations are shown in Table II. The results for a number of these samples, most of which are from Akron, are not seriously subnormal but are included to complete the picture indicating the degree of subnormality of mixing time associated with subnormal loaf volumes. The loaf volumes considered subnormal are italicized.

Out of the total of 505 hard winter wheat samples tested, there were no others which were characterized by the extreme degree of mixing time and loaf volume subnormality typical of many of the samples in Table II. There were, however, several others whose mixing times either approached or were border-line samples of critical subnormality or whose loaf volumes were sufficiently subnormal to warrant attention. The mixing times and loaf volumes for these samples are given in Table III.

TABLE III

OTHER SAMPLES WITH MIXING TIME OR LOAF VOLUME SUFFICIENTLY SUBNORMAL TO WARRANT ATTENTION (ONLY OTHERS IN ADDITION TO THOSE GIVEN IN TABLE II)

Variety	Station	Year	Mixing time		Loaf volume	
			As rec.	Expected ¹	As rec.	Expected ²
Kharkof	Lincoln, Neb.	1940	1 $\frac{5}{8}$	2 $\frac{1}{4}$	1122 ³	1220
Kharkof	Lincoln, Neb.	1941	1 $\frac{1}{2}$	2 $\frac{1}{4}$	1066	1015
Kharkof	Hays, Kans.	1943	1 $\frac{3}{4}$	2 $\frac{1}{4}$	1120	1220
Blackhull	Hays, Kans.	1940	1 $\frac{5}{8}$	2	1022	1125
Blackhull	Lincoln, Neb.	1940	1 $\frac{1}{2}$	2	1078	1125
Blackhull	Amarillo, Tex.	1942	1 $\frac{3}{4}$	2	900	1005
Yogo	Waseca, Minn.	1942	1 $\frac{1}{2}$	2	915	865
Early B. H.	Amarillo, Tex.	1943	1 $\frac{1}{2}$	2	933	948
Chiefkan	Lincoln, Neb.	1940	1 $\frac{7}{8}$	2 $\frac{1}{8}$	830	960
Chiefkan	North Platte, Neb.	1942	1 $\frac{3}{8}$	2 $\frac{1}{8}$	748	800

¹ Average of several years data.

² Expected on basis of protein content of sample and variety regression line.

³ Loaf volumes considered subnormal are italicized.

The mixing times for the samples from North Platte and Colby (Table II) are definitely subnormal compared with those expected from an average of several years data. In addition, all loaf volumes are subnormal except that for Nebred from North Platte. Similarly the mixing times for the samples from Lincoln are subnormal but to a somewhat

less extent for most samples than are those from Colby and North Platte. This is also true for loaf volume. Although the mixing times for all samples from Akron are subnormal, they are only slightly so, and only two loaf volumes are subnormal.

A clearer picture of the extent to which mixing time may be subnormal without resulting in subnormal loaf volume is shown in Fig. 7.

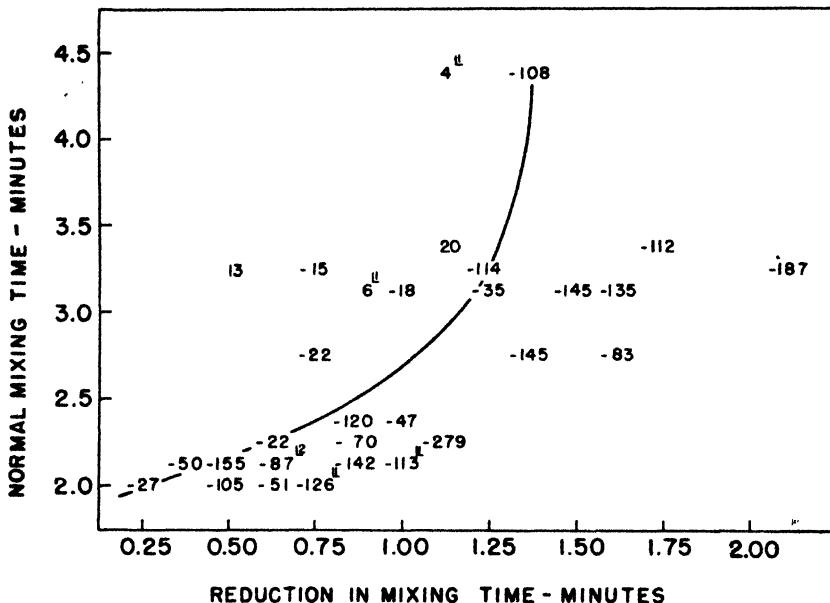


Fig. 7. Loaf volume deviations from normal when mixing times are subnormal (a) by varying amounts, normal mixing time being held constant along the horizontal and (b) by fixed amounts, normal mixing time being increased upward along the vertical. (1 and 2—average of two and three determinations, respectively).

When inspecting the loaf volume deviations along the horizontal of Fig. 7, it will be noted that a reduction in normal mixing time of $\frac{1}{2}$ minute to $1\frac{1}{8}$ minutes for normal mixing times of $3\frac{1}{8}$ to $3\frac{3}{8}$ minutes does not result in loaf volume reductions (Table II). For a reduction greater than $1\frac{1}{8}$ minutes, however, large loaf volume reductions follow. Thus $1\frac{1}{8}$ to $1\frac{1}{4}$ minutes is considered to be the *critical reduction* for a normal mixing time of $3\frac{1}{8}$ to $3\frac{3}{8}$ minutes. As normal mixing time decreases, the *critical reduction* value decreases until at a normal mixing time of 2 minutes it is only about $\frac{1}{4}$ minute, since all mixing times that were below normal by more than $\frac{1}{4}$ minute are associated with loaf volumes that are materially *below* normal. Instead of working along the horizontal, consider the loaf volume deviations on the vertical at fixed reductions in mixing time. For example, a reduction of $\frac{1}{2}$ minute for normal mixing times of 2 and $2\frac{1}{8}$ minutes

resulted in loaf volume reductions of 105 cc. and 155 cc. When the same reduction in mixing time of $\frac{1}{2}$ minute was associated with a normal mixing time of $3\frac{1}{4}$ minutes, however, there was no reduction in loaf volume. Similarly, a reduction in mixing time of $\frac{1}{8}$ minute for a normal mixing time of $2\frac{1}{8}$ to $2\frac{3}{8}$ minutes is associated with an average loaf volume reduction of over 100 cc., but for a longer normal mixing time of $3\frac{1}{8}$ minutes does not result in a loaf volume reduction.

The curve in Fig. 7 has been drawn so that all loaf volume deviations accounted for by experimental error are appreciably above or to the left of the curve; whereas all those representing varying degrees of significance are below or to the right. This curve can be used to estimate the critical mixing times for known varieties of wheat having different normal mixing times. Thus for variety samples that normally have mixing times of from 2 to about $2\frac{1}{2}$ minutes, $1\frac{5}{8}$ ($2\frac{1}{4}$ – $\frac{5}{8}$) is the critical mixing time below which loaf volume likely will be subnormal. For mixing times above $2\frac{1}{2}$ minutes, however, this critical point increases as normal mixing time increases. Thus mixing times of about 2 minutes for Tenmarq and 3 minutes for Cheyenne represent critical points.

The only other samples which were characterized by either a marked subnormal loaf volume or mixing time approaching or equal to that considered as critical are given with their mixing times and loaf volumes, both as received and expected, in Table III. The mixing times for 6 of the 10 samples given in Table III have mixing times of $1\frac{1}{2}$ to $1\frac{5}{8}$ minutes and are therefore border-line cases with respect to being critical. Thus the corresponding loaf volume could be either normal or subnormal without introducing any inconsistencies. Of these six samples, the loaf volumes for Kharkof and Blackhull from Lincoln, Nebraska, in 1940, and Blackhull from Hays, Kansas, in 1940, are probably definitely subnormal on the basis of the errors attributed to baking techniques, yeast variability, and experimental milling. Similarly, the loaf volumes for three of the four remaining samples, Kharkof from Hays, Kansas, in 1943, Blackhull from Amarillo, Texas, in 1942, and Chiekan from Lincoln, Nebraska, in 1940, are subnormal; whereas their mixing times only approach critical values.

It is pertinent to point out that during the drought years in the hard winter wheat area, particularly in 1935 and 1936, the mixing times for all varieties were greatly increased, thus indicating that the protein was wetted with much more difficulty when dehydrated to an excessive degree. Thus the somewhat above-critical mixing times for Kharkof from Hays and Blackhull from Amarillo (Table III) may be the result of drought conditions such as are known to have prevailed during the growing of the 1943 crop at Hays. The temperatures during the fruit-

ing period (studies to be reported in a later publication) were sufficiently high to account for the subnormal loaf volumes of these two samples.

These data suggest that the factor causing subnormal mixing times also will result in poorer protein quality as reflected in lower loaf volumes, provided it operates to the extent of producing sufficiently subnormal mixing times. The property of mixing time is regarded as being associated with or related to the physical and/or chemical constitution of the protein of any given variety. It appears, therefore, that there is a critical mixing time, characteristic of each variety, below which the physical constitution of the protein is definitely different from that characterized by the longer but still subnormal mixing requirements. In addition, the loss of normal elasticity and gas-retaining properties associated with subnormal loaf volumes indicates that the protein structure has been weakened. The fact that variety flours with subnormal loaf volumes almost invariably have critically subnormal mixing times appears to justify the use of mixing time as a means of deciding which samples are satisfactory for quality testing in the same way that test weight is now used.

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COMPARISON OF CORN STARCHES AT VARIOUS STAGES OF KERNEL MATURITY¹

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ABSTRACT

The starch content in the grain of Iowa Hybrid 939, Iowax 1, and Golden Cross Bantam varieties of corn increased in the interval from 12 or 13 days after pollination to maturity. The most rapid increase occurred in the period from 12 to 20 days after pollination. The average of the starch granule diameter in the period from 13 days after pollination to grain maturity rose from 2.7 to 9.9 μ in the nonwaxy dent corn, and from 3.0 to 9.1 μ in the waxy corn. In the sweet corn, the corresponding values for the simple granules were 1.8 to 3.6 μ . The water-binding capacity of the starches at 12 to 13 days was approximately 0.9 g. per g. of starch and diminished rapidly to about 0.3 g. per g. at 20 to 36 days. Over the period studied, the iodine-sorptive capacity of the nonwaxy corn starches increased from 10 to 14 mg. per g. to 54 mg. per g. A low amylose content in starch from immature corn was verified by fractionation of starch from sweet corn 12 days after pollination. Two series of curves were obtained for Iowa Hybrid 939 and Iowax 1 corn starches, respectively, by plotting light transmittancy against temperature as dispersions of the starches were heated. Simple sigmoid curves were obtained for all samples of both series of starches except the mature, nonwaxy, dent corn starch. The latter gave a transmittancy curve with two inflections.

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Reports on the rate of formation, granule size, amylose to amylopectin ratio, and other characteristics of starch during the maturation of the corn kernel are quite limited. Evans (9) prepared photomicrographs of granules of yellow field corn starch at four stages of development to show the increase in diameter and the change from circular to polygonal outline of the granules with increase in kernel maturity. Hixon *et al.* (14) found that the viscosity of starch from corn at early stages of maturity was low and that it increased sharply at 4 to 5 weeks after pollination of the corn.

During the past several years considerable quantities of immature corn have reached the industrial market. Weather conditions unfavorable for maturing the crop and the practice of planting corn which requires a long growing period were major factors leading to this result. The effect of granule size upon processing yield and the suitability for industrial use of starch from immature corn have, consequently, made further knowledge of such starch a matter of practical significance.

Investigations were undertaken, therefore, to determine the approximate rate of starch formation in corn endosperm and some comparative physical and chemical properties of the starch granules at several stages of maturity of the corn.

Materials and Methods

Iowa Hybrid 939 dent corn, Iowax 1 hybrid waxy dent corn, and a sweet corn variety, Golden Cross Bantam, were selected for study. Plantings were made in plots at the laboratory during three growing seasons, but work was confined chiefly to samples grown in 1945 and 1946.

The corn was pollinated by hand and samplings were made at various times from 12 to 13 days after pollination to maturity. Since about 24 hours are required for the pollen tube to reach the embryo sac, fertilization occurred about 1 day after pollination.

The ears of corn were collected between 8:00 and 9:00 a.m. and shelled immediately. Kernels of 12- and 13-day corn were frozen on solid carbon dioxide after shelling. At all other immature stages, the entire ears were frozen on solid carbon dioxide before shelling. All immature samples were kept frozen until they were processed for starch.

Moisture in the kernels was determined by drying a sample overnight in a vacuum oven at 60° to 70°C., grinding to a fine powder, and drying aliquots in a vacuum oven for 6 hours at 100°C. Moisture in the starch was determined by drying *in vacuo* in an Abderhalden drier for 4 hours at 100°C.

Nitrogen was determined by a modification of the Kjeldahl-Gunning-Arnold method (1) in which the ammonia was distilled into a boric acid solution (20).

Starch was determined polarimetrically in a calcium chloride extract of the dry, ground corn (8). Starch was separated from the samples of the 1945 crop year by the second method described by MacMasters and Hilbert (15). The starch obtained by this method was low in total nitrogen, but part of the small-granule fraction was lost in discarding the gluten layer.

In 1946 and 1947 a method was used by which an essentially quantitative separation was made of the starch from the kernel. The early samples, through 35 days after pollination, required no steeping. Mature samples of Iowa Hybrid 939 variety corn were steeped in distilled water for 3 days at 49°C. The starch granules showed no microscopic evidence of enzymic attack. Corresponding samples of waxy and sweet corn were steeped for 45 to 48 hours in a solution having an initial sulfur dioxide concentration of 0.2%.

Disintegration of the kernels was effected in iced distilled water (7 to 8 ml. per g. of corn) in a high-speed blender. The fine material was separated by passing it through No. 17 standard silk bolting cloth. The residual wet pulp of the mature samples, which still contained much starch, was passed through a hammer mill. The pulp of Iowa Hybrid 939 variety and sweet corn was first steeped overnight in potassium hydroxide solution at pH 10.5 to 11.5. The ground material was washed on No. 17 standard silk bolting cloth.

The combined washings were centrifuged and when a lower layer of pure starch had formed it was separated from the upper layer of small starch granules, protein, and fiber. This upper layer, or in some cases the entire sample, was treated two or three times alternately with 80% ethanol and potassium hydroxide solution at pH 10.5 to 11.5 to remove protein. Fiber was separated from the starch by gravity sedimentation and by washing on No. 17 standard silk bolting cloth. The starch so obtained was combined with any starch originally obtained by centrifuging. Microscopic examination of the fiber fractions showed negligible loss of starch. After correcting for protein and moisture, the weight of starch recovered by this method was taken as a measure of the starch content of the grain. Protein was calculated by multiplying the total nitrogen by the factor 6.25.

Microscopic measurements of granule size were made only on starch from corn grown in 1946. Each sample for measurement of granule size was drawn with a large-orifice pipette from a suspension in which the starch granules were kept uniformly distributed by

rapid stirring. A drop of the suspension was mixed thoroughly with a drop of glycerol on a microscope slide and covered with a cover slip.

A microscope equipped with an 0.85 N.A. apochromatic objective and a 15X compensating eye piece was used for measuring granule size. Camera lucida drawings were made on graph paper of all granules in 3 to 10 fields, usually more than 5, selected at random from as many slides. Each granule was brought into focus before it was drawn. The magnification of the drawings was determined with the aid of a stage micrometer.

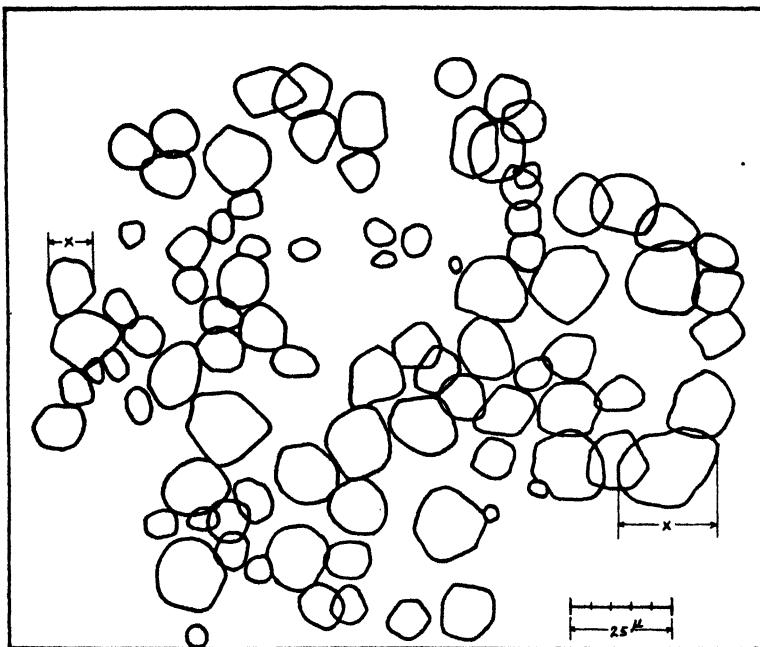


Fig. 1. Camera lucida drawing of a selected field of starch granules from mature Iowa Hybrid 939 dent corn. Granules were measured by their projection on the horizontal axis. $\times 1150$.

Statistical diameters (6) were determined by measurement of the drawings. Because of the great spread in granule size, samples of from 530 to 2,483 granules were used. A selected field illustrating the method of granule diameter measurements is given in Fig. 1. The size frequency distribution of the starch granules shown here roughly approximates that of the mature starch of Iowa Hybrid 939 dent corn. The frequency distribution curve derived from a sample of 959 starch granules of this variety is shown in Fig. 3.

The water bound by the freshly prepared, undried starch before treatment with ethanol and potassium hydroxide was determined by the refractometric method of Dumanski (7). The method is based on

the hypothesis that bound water does not act as a solvent for dissolved solutes. The starch sample was mixed with a sucrose solution of known concentration. After the water in the system had attained an equilibrium distribution with respect to the water bound by the starch and that acting as solvent for sucrose, the change in sucrose concentration was determined refractometrically. The water bound by starch in this equilibrium mixture was calculated by Dumanski's equation for samples free of solutes,

$$X = \frac{ap}{100} + B \frac{b_2 - b_1}{b_2},$$

where X = grams of bound water in p grams of original sample,

a = per cent of water in sample (determined by drying *in vacuo* at 100°C),

p = weight of sample in grams,

B = grams of original sucrose solution,

b_1 = per cent of sucrose in original solution,

b_2 = per cent of sucrose in equilibrium solution.

Any effect on the bound-water content of the samples due to protein contamination was not taken into account in calculating the water bound by the starch.

Only starch separated from kernels of the 1946 crop by the quantitative method was used. From 0.1 to 2.1 g. (dry basis) of starch were shaken with 4 to 12 g. of 10 to 60% sucrose solution for 30 minutes. The suspension was centrifuged to remove the starch prior to determination of the refractive index of the equilibrium solution. In 60% sucrose solution, the specific gravity was sufficiently high that the starch did not sediment and filtration was required. In general, the values found for the bound-water content of starch decreased as the sucrose concentration of the equilibrium solution was increased.

Starch was defatted prior to fractionation and to determination of iodine-sorptive capacity by extraction for 48 hours in a Soxhlet- or Butt-type extractor with 85% methanol in the extraction flask.

As a measurement of the amylose content, the iodine-sorptive capacity of the defatted starches was determined by the method of Bates, French, and Rundle (2), as modified by Wilson, Schoch, and Hudson (19). Determinations were made on immature Iowa Hybrid 939 and sweet corn starches both before and after the separation of most of the contaminating protein. There was no interference with the determination by as much as 20% protein in the sample.

Starch was fractionated by a modification (cf. 13, J. E. Hodge, unpublished data) of Schoch's butanol precipitation method (17), but

the crude amylose was autoclaved only 10 to 15 minutes in the purification procedure. The purification process was repeated until the iodine-sorptive capacity of two successive precipitates agreed within 1%. The amylopectin solution was concentrated under reduced pressure, below 50°C., and the amylopectin was precipitated from the aqueous concentrate by adding it slowly to absolute ethanol. It was then dried in a vacuum desiccator over calcium chloride, after which drying was completed in a vacuum oven at 70°C.

The light transmittancy of starches during pasting in water was followed by the method of Morgan (16) with apparatus modified by S. A. Karjala (unpublished). Light transmittancy data were obtained only for the starches prepared in the 1945 season.

Results and Discussion

Moisture content of the corn kernels decreased from about 87% at 12 to 13 days after pollination to 9 to 11% at maturity (Table I).

TABLE I
AGE AND MOISTURE CONTENT OF GRAIN

Variety	1946	
	Age, ¹ days	Moisture content, %
Iowa Hybrid 939 dent corn	13	86.8
	15	86.2
	20	78.9
	35	55.6
	Mature ²	11.2
Iowax 1 hybrid waxy dent corn	13	86.4
	15	84.5
	20	78.4
	35	61.3
	Mature ²	10.9
Golden Cross Bantam variety sweet corn	12	86.5
	14	80.5
	20	76.3
	35	60.9
	Mature ²	9.0

¹ Age is defined as the number of days after pollination.

² Mature corn samples were harvested 70 to 74 days after pollination.

Over the same period, the amount of starch in the corn increased (Fig. 2). The rise in starch content in all three varieties was most rapid between 12 and 20 days after pollination. All of the varieties of corn studied contained less starch than was found by Evans (10) in his study of the maturation of Minnesota Hybrid 403 dent corn.

The polarimetric method of analysis indicated higher starch content at most stages of maturity than was obtained by separation (Fig. 2). Part of this difference resulted from a slight loss of starch during

recovery, but most of it is attributable to the fact that the polarimetric procedure measures both starch and soluble polysaccharides. This was especially evident in sweet corn in which less than 50% of the total polysaccharides was recovered as starch. The yield of 26% starch obtained from mature Golden Cross Bantam sweet corn was in good agreement with the 21 to 25% starch content of Golden Bantam sweet corn reported by Sumner and Somers (18).

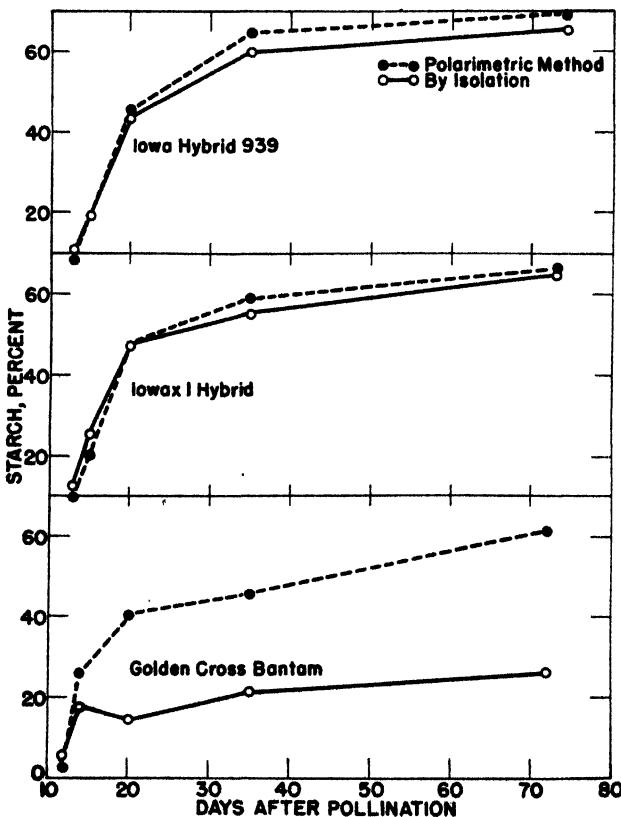


Fig. 2. Starch content of corn at different stages of kernel maturity (dry basis).

The total nitrogen content of the starches prepared by the quantitative procedure was high in comparison with that of starches prepared by the method in which part of the small granules were lost into the tailings fraction (Table II).

Two types of series of granule size distribution curves were obtained (Fig. 3). Beginning with a narrow range of granule diameters at 13 days after pollination, the granule size frequency distribution curves of the two dent corn starch varieties showed a marked spread and a

rapid shift of the peak as the corn increased in maturity. The sweet corn starch curves, on the other hand, showed little change in spread or in the position of the peak. This difference is attributable to the fact that in the varieties studied the dent corns contain simple starch granules which vary considerably in size, while the sweet corn starch

TABLE II
IODINE-SORPTIVE CAPACITY AND TOTAL NITROGEN CONTENT OF STARCH

Variety	Time of sepn. of starch, days after pollination	Iodine-sorptive capacity, mg. per g. starch (d.b.)		Total N, % (d.b.)	
		1945	1946	1945	1946
Iowa Hybrid 939 dent	13	—	13	—	1.4
	15	29	22	0.04	1.2; ¹ 0.28
	18	40	—	0.03	—
	20	—	41	—	1.1; ¹ 0.24
	21	42	—	0.03	—
	35	—	50	—	0.91;0.20
Iowax 1 hybrid dent	Mature	48	53	0.06	0.42
	13	—	5.4	—	1.13
	15	0.8	2.9	0.01	0.97
	18	0.8	—	0.02	—
	21	0.7	—	0.02	—
	Mature	0.9	2.5	0.04	0.29
Golden Cross Bantam sweet		1946	1947	1946	1947
	12	10	24	—	0.15
	14	28	—	1.7;0.85	—
	20	32	—	3.5;1.9	—
	35	54	—	3.2;1.1	—
	Mature	53	—	0.29	—

¹ Values before and after treatment, respectively, with 80% ethanol and KOH solution at pH 10.5 to 11.5. These starches were prepared by the quantitative method.

granules are chiefly compound. These compound granules break during processing into simple granules which are relatively uniform in size. Results on the sweet corn starch at 20 days after pollination are out of line, probably because this sample came from corn that was pollinated and harvested out of sequence with the other corn samples.

The average granule diameter of the dent corn starches increased most rapidly over the period 13 to 20 days after pollination, after which the increase was slower (Fig. 4). For sweet corn starch, the rate of increase of average granule diameter was less than for the dent corn starches, and was approximately linear over the entire period studied.

Starch granules from mature Iowa Hybrid 939, Iowax 1, and Golden Cross Bantam varieties were found to have average diameters of 9.9, 9.1, and 3.6μ , respectively.

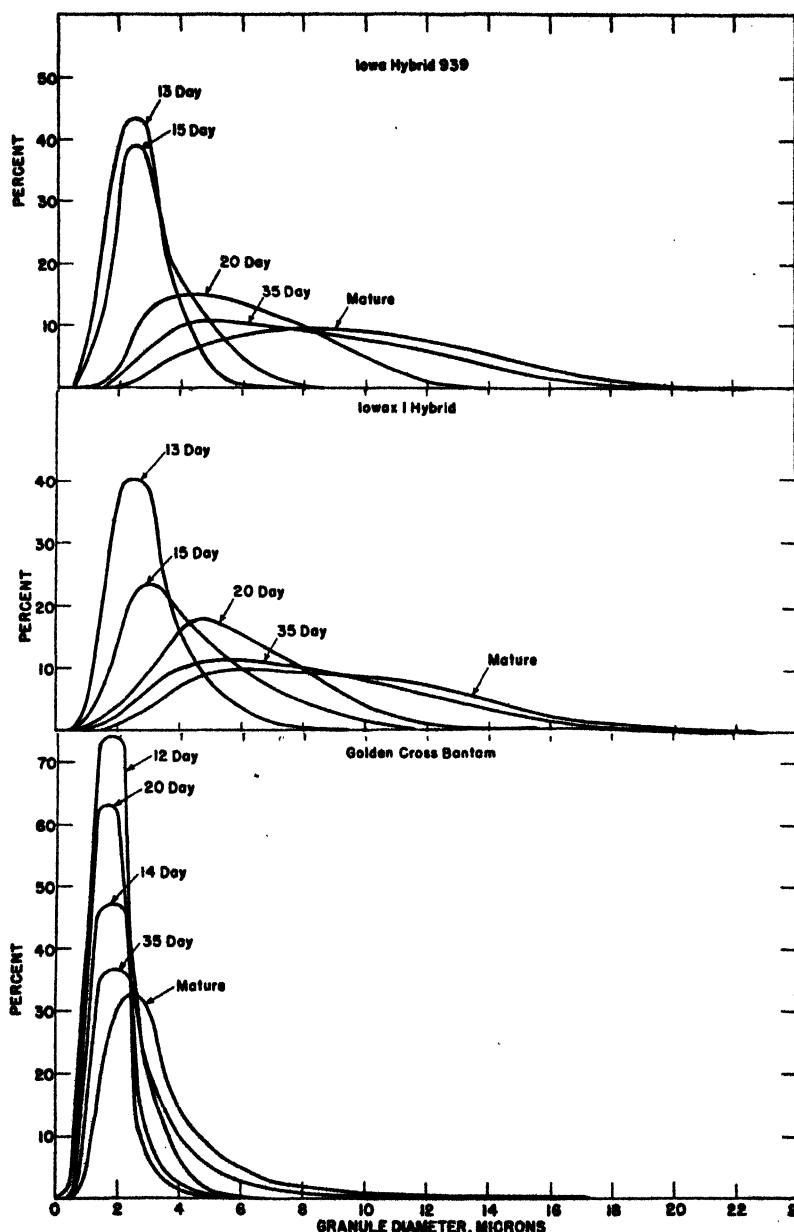


Fig. 3. Size frequency distribution curves of starch granules at different stages of kernel maturity. The curves were smoothed from histograms with an interval of 1μ .

The water-binding capacity of the three varieties of corn starch granules in equilibrium with 10 to 60% sucrose solution diminished rapidly over the period 12 to 20 days after pollination. The water-binding capacity during this period decreased to a greater extent than

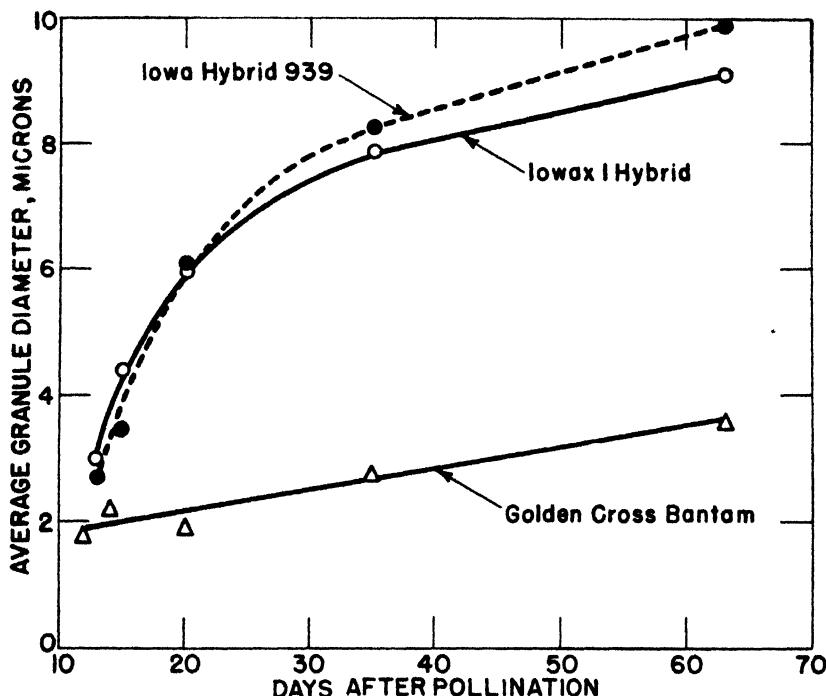


Fig. 4. Average starch granule diameters at different stages of kernel maturity.

did the moisture content of the kernel (Table I). Typical data (Fig. 5) showed that in equilibrium with 10% sucrose solution the starch bound about 0.9 g. of water per g. at 12 to 13 days after pollination. At 20 days, the corresponding value was approximately 0.3 g. per g. Chesheva (5) reported approximately 0.12 g. of water bound per g. of corn starch, from mature corn, in equilibrium with 10% glucose solution. Potato starch was found by Freeman (11) to bind an average of 0.31 g. of water per g. in equilibrium with sucrose solutions of different concentrations.

Starch from immature corn of the nonwaxy varieties had a lower iodine-sorptive capacity, indicating a lower ratio of amylose to amylopectin, than the corresponding starch from mature corn. Waxy corn starch had the anticipated low iodine-sorptive capacity, characteristic of the absence of amylose, throughout the maturation period of the corn (Table II). From 12 to 13 days after pollination to maturity, the

iodine-sorptive capacity of sweet corn starch increased by approximately a factor of five, while that of nonwaxy dent corn starch increased about fourfold.

Fractionation of starch from sweet corn 12 days after pollination yielded 10.5% amylose, in good agreement with the 12% amylose content calculated from the iodine-sorptive capacity (24 mg. per g.) of the sample. Iodine-sorptive capacity of the amylose fraction reached a limiting value of 200 mg. of iodine per g. The amylopectin fraction sorbed 9.3 mg. of iodine per g.

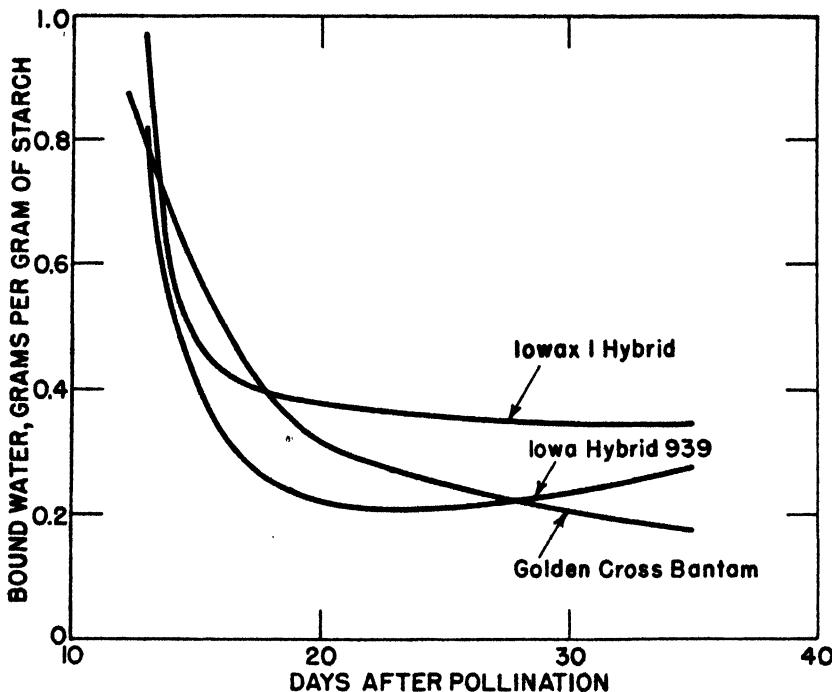


Fig. 5. Bound water content, on a dry weight basis, of corn starches at different stages of kernel maturity, in equilibrium with 10% sucrose solution.

There were appreciable differences in iodine-sorptive capacity of comparable starches separated from corn grown in different years (Table II). These differences cannot be explained on the basis of present knowledge.

Current information concerning the enzymic synthesis of starch is not sufficient to afford a clear explanation for a progressive increase in the proportion of linear molecules in starch as the grain matures. Bourne and Peat (4) considered at least two enzymes, P and Q, to be involved in the conversion of glucose-1-phosphate to a product similar

to amylopectin. Phosphorylase (the P enzyme) and glucose-1-phosphate yield a linear glucose polymer. The Q enzyme is believed to catalyze the formation of amylopectin from amylose through the hypothetical intermediate, "pseudoamylose." Bourne and Peat expressed the opinion that the ratio of the P and Q enzymes determines whether amylose, amylopectin, or a mixture of the two will be produced.

If a linear polymer is the precursor of both amylose and amylopectin, the enzyme system must favor further polymerization to a non-linear molecule more in the immature grain than it does in grain nearing maturity. Possibly there is circumstantial evidence for this view in the relatively large amount of water-soluble polysaccharide, apparently similar to glycogen (12), present in immature sweet corn. Starch granules lie embedded within globules of this material, which suggests that this water-soluble polysaccharide may be an intermediate in starch formation.

Morgan (16) noted that suspensions of starch with large granules transmit more light during the pregelatinization period than suspensions with small granules. It was recently reported by Beckord and Sandstedt (3) that the light transmittancy of the large-granule fraction of wheat starch is greater than that of the small-granule fraction over the entire heating range. The granule size frequency distribution curves of Fig. 3 do not apply to the corn starches which were prepared in 1945, because the small granules were not adequately represented. Despite this fact, there is sufficient variation in granule size at the different stages of maturity to show clearly the dependence of light transmittancy on the granule size. Waxy corn starches gave simple sigmoid curves (Fig. 6) which showed an increase in light transmittancy over the entire temperature range with increase in maturity of the grain. Nonwaxy dent corn starches showed a correlation between light transmittancy and granule size only in the pregelatinization range. Only the starches from immature kernels of this series gave curves which were sigmoid in character. At maturity the curve showed two inflections with a region of relatively low light transmission between 75° and 90°C.

The most rapid changes in starch properties were found to occur during the first 35 days after pollination. Changes in water-binding capacity and iodine-sorptive capacity which occurred after this period were probably too small to be of significant effect upon industrial processing of corn and utilization of the starch. From the standpoint of commercial starch production, however, changes in starch content of the grain and in granule size of the starch might be of significance to the processing industry. In Iowa Hybrid 939 corn, for example, the

starch content was approximately 10% less at 35 days after pollination than at maturity. At the earlier date about 66% of the granules were less than 10 μ in diameter, while in mature corn 53% of the granules were in this range

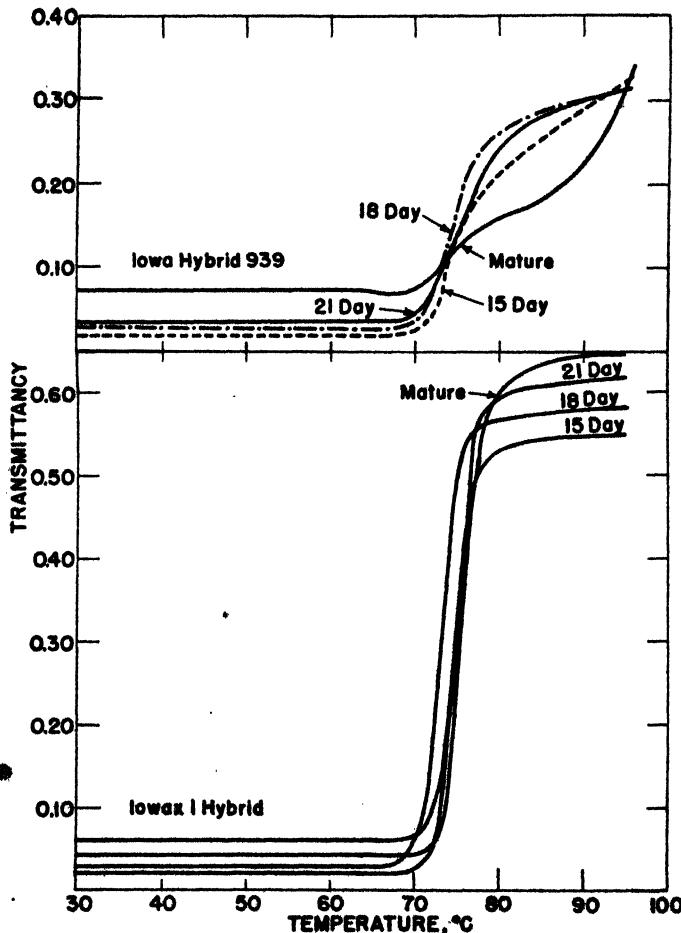


Fig. 6. Light transmittancy curves of 0.1% suspensions of corn starches at different stages of kernel maturity.

Although small granules are in the majority on a number basis, calculations on a weight basis, assuming an average density of 1.63 for corn starch, show the minor contribution of the small granules to the total weight of the starch. The granules having diameters up to 10 μ account for 21% of the weight of the starch at 35 days after pollination and 15% of the weight of the starch at maturity. Thus it is apparent that since the smaller granules are the most difficult to

recover in processing, the loss of starch in commercial processing would be greater with immature corn.

Acknowledgment

We are indebted to F. R. Earle and others of the Analytical and Physical Chemical Division of this laboratory for the analyses for moisture and starch in corn. The assistance of Carol M. Jaeger in the statistical analyses and of Margaret N. Mason, John A. Cannon, Beatrice Jacobs, Margaret Holzapfel, John Warner, and General Irving in the laboratory work is gratefully acknowledged.

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INFLUENCE OF PROTEOLYTIC ENZYMES AND YEAST NUTRIENTS UPON THE REQUIREMENT FOR MALT IN GRAIN ALCOHOL FERMENTATIONS¹

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ABSTRACT

The value of proteolytic enzymes, protein hydrolyzates, vitamins and other growth factors for yeast as partial replacements for barley malt in grain alcohol fermentation was determined. Slurries of corn or wheat were premalted at 70°C. for 30 minutes with malt equivalent to 1% of the grain bill, after which mashes were cooked at 25-lbs. pressure for 30 minutes. The cooked mashes were cooled and varying quantities of conversion malt and adjuncts were added. After holding at 58°-60°C. for 30 minutes the temperature was lowered to 30°C., and 2% by volume of distillers' yeast NRRL No. Y-567 was added. The action of adjuncts upon both the rate of fermentation and the final yield of alcohol was followed.

Papain, ficin, culture filtrates from *Bacillus subtilis*, and protein hydrolyzates were found capable of substituting for a portion of the conversion malt. When these were supplied at their optimum concentration equivalent yields of alcohol and rapid fermentation rates were obtained with 60% of the normal amount of malt. Some beneficial effect was noted by adding the same materials to yeast seed cultures. It is shown that the effect of proteolytic enzymes is associated with the nutrition of the yeast rather than with the release or activation of malt amylases.

To meet urgent wartime demands, industrial alcohol was manufactured from 1943 through 1945 at a rate about fivefold over prewar production. While fermentation of molasses and synthesis from ethylene provided a portion of our wartime alcohol requirement, the bulk of it was supplied by processing grain and grain products (1). This increase in the utilization of grain required correspondingly greater quantities of barley malt, and for a time curtailment of grain alcohol production was imminent owing to a shortage of distillers' malt.

In view of this contingency, an investigation was undertaken to determine whether the amount of barely malt, which normally comprises 8 to 10% of the grain entering the distillery, could be reduced without sacrificing the yield of alcohol or unduly prolonging the fermentation time. The present study is concerned with the effects of proteolytic enzymes and yeast nutrients upon the malt requirement in corn and wheat mashes used for alcohol production.

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The liberation or activation of bound amylases by proteolytic enzymes has long been known, and this property is commonly utilized in determining the total amylase activity of cereals (Myrback and Myrback (5), Snider (8)). Likewise, numerous investigators have recognized the stimulatory properties of protein degradation products upon yeast growth. These studies recently reviewed (Thorne (10)) have revealed that available ammonium salts, asparagin, aspartic acid, glutamic acid, leucine, and arginine are outstanding in their stimulatory action. The remaining amino acids vary appreciably in the response which they elicit with a few actually being inhibitory. With regard to peptides, availability to the yeast cell decreases as the length of peptide chain increases with the result that proteins and high molecular weight peptides are virtually without effect. The nitrogen nutrition of yeasts has been examined largely through the use of synthetic and grain wort media, and the knowledge gained therefrom has not been applied extensively to alcoholic fermentation of whole grain mashes.

Methods

For most of these experiments, grain mashes were prepared by weighing 45.0 grams of ground corn or wheat and 0.5 gram of barley malt into 500 ml. flasks and adding 170 ml. of water at 70°C. to each flask. The flasks were placed in a water bath at 70°C. and the grain slurries were stirred intermittently during 30 minutes. Following this premalting, mashes were cooked in the autoclave for 30 minutes at a steam pressure of 25 pounds. After cooking, mashes were cooled to 70°C. and the malt, slurried with 50 ml. of water, was added. The flasks were then placed in a water bath adjusted to 58-60° and conversion was continued for 30 minutes during which the mashes were agitated frequently. With mashes saccharified with malt plus adjunct where less conversion malt was used, a correspondingly larger quantity of corn or wheat was mashed. Converted mashes were cooled to 30°C. and inoculated with 2% by volume of a 24-hour culture of distillers' yeast NRRL strain No. Y-567. The final volume in each flask was approximately 250 ml.

Fermentation was conducted at 30°C. Progress was followed either by weighing the flasks or determining their alcohol content. At 72 hours, unless otherwise noted, the beers were brought to a volume of 300 ml. and a 200-ml. portion was taken for determination of alcohol. One hundred ml. of distillate was collected from the 200 ml. of beer and the alcohol concentration in the distillate was measured by reading the refractive index with a dipping refractometer. Data reported are in all cases the averages of duplicate flasks.

Alcohol yields are expressed as proof gallons per 56 pounds of total grain as used. The wheat employed had the following percentage composition: Moisture, 11.6; starch (A.O.A.C.), 59.4; sugars as glucose, 1.9; pentosans, 5.6; and nitrogen, 2.06. The percentage composition of the corn was: Moisture, 10.5; starch (polarimetric), 63.6; sugar as glucose, 1.77; and nitrogen, 1.49. The barley malt was a commercial sample with 180° Lintner value. Its percentage composition was: Moisture, 8.05; starch (polarimetric), 40.7; sugar as glucose, 9.11; and nitrogen, 2.22.

Experimental

Papain in Wheat Mashes. Wheat mashes prepared as described, except for the use of larger volumes to permit frequent sampling, were converted with (a) 8% malt, (b) 5% malt, (c) 5% malt plus varying levels of papain, and (d) 5% malt plus similar levels of heat-inactivated papain. After conversion and cooling, the mashes were inoculated and allowed to ferment 50 hours. Fermentation progress was followed by periodically determining the alcohol content of the mashes.

TABLE I

THE EFFECT OF ADDING PAPAIN AND HEAT-INACTIVATED PAPAIN TO WHEAT MASHES ON THE RATE OF FERMENTATION AND YIELD OF ALCOHOL

Papain added	Malt added	Alcohol production				Final yield
		Percent by volume after				
Mg./100 ml.	Percent of grain	18 hours	40 hours	50 hours	Proof gal./bu.	
Untreated papain						
None	8	3.50	7.14	7.30	4.84	
None	5	2.70	6.04	6.57	4.41	
1	5	3.47	6.82	7.03	4.72	
10	5	5.38	7.07	7.17	4.82	
20	5	5.49	7.07	7.31	4.92	
50	5	5.68	7.04	7.31	4.92	
100	5	5.74	7.10	7.20	4.83	
Heat-inactivated papain¹						
1	5	2.74	6.04	6.70	4.51	
10	5	2.66	6.08	6.67	4.47	
20	5	2.66	6.15	6.68	4.48	
50	5	2.74	6.00	6.56	4.41	
100	5	3.03	6.36	6.90	4.63	

¹ Papain was inactivated by autoclaving at 121°C. for 15 minutes.

Results presented in Table I show clearly that in mashes receiving papain, fermentation started much earlier than in the controls which were converted with either 5 or 8% malt. After 18 hours the alcohol concentration in papain mashes was approximately double that in the

5% malt control. While the difference became less pronounced as fermentation continued, nevertheless, upon final analysis mashes converted with 5% malt plus either 20 or 50 mg. % papain gave yields equivalent or slightly higher than the 8% malt control. Those receiving 10 and 100 mg. % papain were nearly equivalent to the 8% malt control while the addition of as little as 1 mg. % papain resulted in a significant improvement over the 5% malt control. With heat-inactivated papain there was little or no stimulation of fermentation, except for a small increase with the highest level, due possibly to the presence of heat-stable nutrients contained in the enzyme preparation.

Stimulation by Other Proteolytic Enzymes. The action of ficin and the proteinase from *Bacillus subtilis* (scaber strain NRRL B-544) on the fermentation of grain mashes is shown in Table II. Proteinase production by this organism cultured on the surface of asparagus butt medium has been described by Kline *et al.* (4). In preliminary experiments it was found that very active proteolytic preparations could be produced by growing the organism under submerged aerobic culture conditions in a medium composed of thin grain stillage, glucose, and calcium carbonate. The proteolytic culture liquor used in this experiment was obtained by cultivation of *B. subtilis* NRRL B-544 for 72 hours in thin grain stillage supplemented with 2% glucose and 1% calcium carbonate. This culture liquor was filtered but no attempt was made to effect complete removal of the bacteria.

Duplicate flasks of corn mash, after cooking and cooling, were converted with the following preparations: 10% malt; 4% malt; 4% malt plus papain; 4% malt plus ficin; 4% malt plus two levels of *B. subtilis* culture filtrate; and 4% malt plus untreated thin grain stillage.

Weight losses (Table II) show an increased rate of fermentation in mashes which received the culture liquor, papain, and ficin; the differences again were especially apparent after 18 and 27 hours. With continued incubation, the control mashes approached the enzyme-treated mashes with respect to their total loss in weight. Final alcohol yields after 66 hours of incubation showed that the papain and ficin-treated mashes, although considerably improved in fermentation efficiency over 4% malt mashes, were slightly lower than those in which 10% of malt was used. Mashes supplied with *B. subtilis* culture filtrate, however, were at least equivalent to those employing the high level of malt. Thin stillage slightly increased the rate of fermentation but did not significantly raise the final alcohol concentration. All mashes receiving adjuncts were less acid after fermentation. This condition invariably prevailed in proteolyzed mashes. It seemingly is caused either by the increased buffer action of the protein degradation products or by the more rapid utilization of sugar by the yeast, thus sup-

pressing growth and acid production by bacteria introduced with the malt.

Repeated trials wherein suboptimal levels of malt were supplemented with proteinases resulted in all cases in a shortened lag period and an increased rate of fermentation. The yield of alcohol was either equivalent or superior to that produced by 8% malt. These results indicated that proteinases were either liberating the malt amylases more completely, or they were supplying other substances which en-

TABLE II

THE EFFECT OF BACTERIAL AND PLANT PROTEINASES ON THE RATE OF FERMENTATION AND THE YIELD OF ALCOHOL FROM CORN MASHES CONVERTED WITH A SUBOPTIMAL LEVEL OF MALT

Malt added percent	Adjunct	Weight loss in grams				Final pH	Yield of alcohol Proof gal./bu.
		18 hours	27 hours	50 hours	66 hours		
10	None	7.2	10.7	16.4	17.2	4.2	5.05
4	None	5.2	7.6	14.5	16.0	4.1	4.70
4	Thin stillage (8% of mash volume)	5.8	8.4	15.0	16.2	4.3	4.74
4	Papain (10 mg. %)	11.4	13.5	15.5	16.5	4.5	4.91
4	Ficin (5 mg. %)	12.7	13.8	15.5	16.4	4.6	4.97
4	<i>Bacillus subtilis</i> (4% of mash volume)	13.4	14.4	16.2	17.2	4.8	5.03
4	<i>Bacillus subtilis</i> (8% of mash volume)	13.5	14.6	16.2	17.1	4.7	5.14

hanced fermentation or saccharification, or both. A study was made, therefore, to determine whether the beneficial action of papain was attributable to its ability to release amylases or to provide yeast nutrients or whether both of these factors were involved.

Effect of Papain upon Saccharification. Cooked corn mashes in double the quantity described under Methods were converted with: (a) 8% malt, (b) 5% malt, and (c) 5% malt plus 10 mg. % papain. Determinations of reducing sugars calculated as maltose were made before inoculation and at intervals during fermentation. Fermentation progress was also followed in separate pairs of flasks by determination of weight losses. Final alcohol concentration was measured in these flasks after a period of 50 hours. Results are plotted in Fig. 1.

By comparison of the sugar values after conversion, it is apparent that papain did not increase the primary saccharification rate. The sugar concentrations were nearly the same in mashes converted with 5% malt with and without papain, and slightly higher as might be expected with 8% malt. However, from the residual sugar concentrations during fermentation, it can be seen that in papain mashes, active fermentation began much earlier and continued at a more rapid rate

with the result that at 24 hours the sugar was almost completely utilized. Mashes without papain showed a definite lag period and required about 40 hours with 8% malt and about 45 hours with 5% malt to reach the same residual sugar level. The beneficial action of papain, therefore, is clearly associated with fermentation rather than with saccharification. This observation was substantiated by determining the alpha-amylase content of mashes converted in the presence and absence of papain, by a modification of the method of Olson,

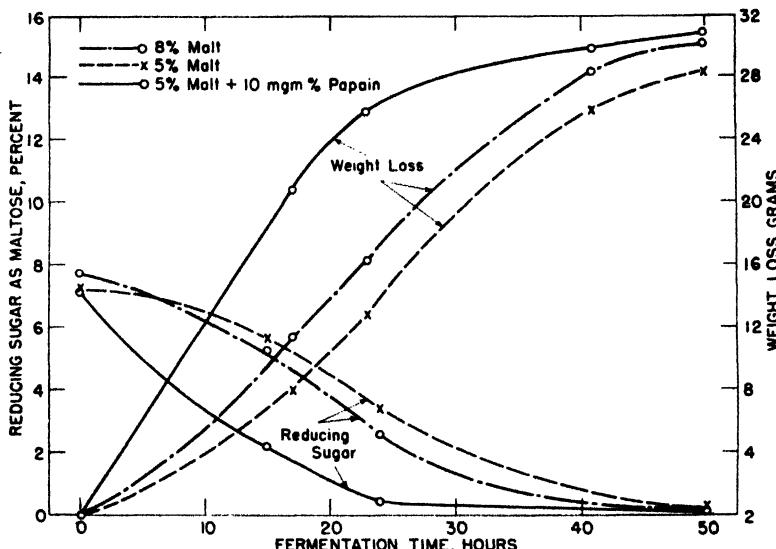


Fig. 1. Progress of alcoholic fermentation of grain mashes with and without papain.

Evans, and Dickson (6). As in the case of reducing sugar, no significant increase in alpha-amylase activity during the conversion period could be demonstrated by adding papain.

Effect of Vitamins. Papain is commonly used either singly or combined with other enzymes to liberate vitamins and growth factors prior to microbiological assay (3). To determine whether the yeast-stimulating action of papain could be explained by its ability to release yeast growth factors, the influence of adding various vitamins and vitamin concentrates was next studied. The following were added to corn mashes saccharified with 5% malt: Thiamin, riboflavin, niacin, pyridoxin, pantothenic acid, biotin, inositol, para-aminobenzoic acid, and a combination of these factors. Also supplied to separate mashes were dehydrated liver extract and dried distillers' solubles. Fermentation rates which were determined by weight losses and final alcohol yields revealed the single vitamins to be ineffective as stimulants and

the combined vitamins to be only slightly effective. Liver extract and dried solubles showed less stimulation than did papain.

Effect of Papain on the Amino Nitrogen Content of Grain Mashes. Two series of mashes were converted with 5% malt, 5% malt plus papain, and 10% malt. One series was inoculated with yeast in the normal manner while the other was incubated at the same temperature without addition of yeast. Malt slurries were pretreated with formalin to suppress bacterial activity. At intervals samples were withdrawn from each series of flasks for the determination of amino nitrogen content by the formol titration method. Results are shown in Table III.

TABLE III

THE INFLUENCE OF PAPAIN ON THE AMINO NITROGEN CONTENT OF CORN MASHES INCUBATED IN THE PRESENCE AND ABSENCE OF YEAST

	Uninoculated mashes			Inoculated mashes		
	5	10	5	5	10	5
Malt concentration, %	None	None	10	None	None	10
Papain added, mg./100 ml.						
Amino nitrogen content, mg./100 ml.						
0 hr.	4.9	5.6	7.0	4.9	5.6	7.0
16 hr.	5.3	5.9	7.2	1.4	2.1	2.5
48 hr.	5.5	6.6	8.5	0.8	1.0	2.0
72 hr.				1.1	1.3	3.7
116 hr.	6.2	7.7	9.8			

It may be seen from these data that there is an increase in the amino nitrogen content of uninoculated mashes during incubation due probably to the proteolytic activity of the barley malt. It is also apparent that papain, as might be anticipated, appreciably increased the amino nitrogen content of mashes saccharified with 5% malt. Such mashes contained even more amino nitrogen than those converted with 10% malt. Of more significance is the fact that most of this nitrogen is available to the yeast, as indicated by the sharp decrease in the amino nitrogen levels in inoculated mashes.

While the amino nitrogen content of papain-treated mashes was not reduced during fermentation to as low a level as in mashes containing only malt, the total utilization of nitrogen was greater in the former. The utilization of such a high proportion of amino nitrogen in all the mashes, and especially in those with suboptimal quantities of malt, suggested that lack of available nitrogen might limit to some extent the activity of the yeast. This possibility was investigated further by adding various quantities of amino nitrogen to mashes converted with insufficient malt.

Effect of Adding Organic Nitrogen. Corn mashes were saccharified with 10% malt, 4% malt, and 4% malt plus varying increments of

acid-hydrolyzed casein, urea, and thin grain stillage. After inoculation, the rate of fermentation and final yields of alcohol were recorded.

Results shown in Table IV demonstrate the complete replaceability of papain with casein hydrolyzate. Although urea produced a similar effect, its use did not result in equivalent alcohol yields and it sharply depressed alcohol formation when used in greater quantities. Casein hydrolyzate, when added to supply from 12 to 24 mg. of amino nitrogen per 100 ml. of mash, stimulated fermentation similar to papain added

TABLE IV

THE EFFECT OF CASEIN HYDROLYZATE, UREA, AND STILLAGE ON THE FERMENTATION OF CORN MASHES SACCHARIFIED WITH A SUBOPTIMAL LEVEL OF MALT

Conversion malt	Nitrogen supplement per 100 ml. of mash	Weight loss in grams			Final pH	Yield of alcohol Proof gal./bu.
		18 hours	42 hours	68 hours		
10	None	7.5	15.5	16.7	4.3	5.17
4	None	5.6	13.6	15.9	4.1	4.76
4	20 ml. stillage	7.5	13.8	16.2	4.2	4.92
4	10 mg. papain	11.4	15.3	16.8	4.5	5.10
Casein hydrolyzate-Amino nitrogen equivalents						
4	2 mg.	7.1	14.4	16.2	4.3	5.04
4	6 mg.	9.2	14.6	16.3	4.4	5.13
4	12 mg.	10.7	14.5	16.4	4.5	5.14
4	24 mg.	12.2	15.4	16.5	4.6	5.20
4	40 mg.	12.7	14.7	16.1	4.6	5.05
Urea-Nitrogen equivalents						
4	2 mg.	6.7	12.8	15.8	4.3	4.83
4	6 mg.	8.4	14.3	16.0	4.3	5.03
4	12 mg.	9.3	14.2	16.0	4.4	4.89
4	24 mg.	11.6	14.3	15.9	4.4	4.86
4	40 mg.	12.3	14.1	15.7	4.5	4.68

at 10 mg. %. Furthermore, even when added at the rate of 2 and 6 mg. of amino nitrogen per 100 ml. of mash, casein hydrolyzate increased substantially both the yield of alcohol and the rate of fermentation. The rate of fermentation was raised still more, but the yield of alcohol was decreased, by the addition of the hydrolyzate to supply 40 mg. % amino nitrogen. Amino acids added singly to supply equivalent amounts of nitrogen were not as effective as casein hydrolyzates. With urea, the fermentation rate was increased slightly less than with casein hydrolyzate and the yield of alcohol was correspondingly lower. When added at a rate of 24 or more mg. of nitrogen per 100 ml., urea lowered the yield of alcohol significantly. Stillage at a level of 20% of the mash volume raised the initial rate of fermentation and the yield of alcohol but to a lesser extent than either casein hydrolyzate or urea when the latter were present in optimal concentration.

Papain Added to Seed Yeast Cultures. The absorption of amino nitrogen from grain mashes with resultant stimulation of yeast activity raised a question as to whether the supplementing of seed yeast media would produce an effect similar to that obtained by adding papain. Ten mg. % papain was added at the time of inoculation to clarified wort prepared by saccharifying corn mash with 20% malt. Seed yeasts were propagated for 24 hours at 30°C. in this medium and in the same medium without addition of papain. To avoid the carry-over of nutrients formed by papain the yeast from each wort was separated by centrifugation, washed, and resuspended in an equivalent volume of sterile water.

Results (Table V) show that considerable activation of the seed yeast can be effected by cultivating it in a medium treated with papain.

TABLE V

THE INFLUENCE OF ADDING PAPAIN TO THE YEAST CULTURE MEDIUM UPON THE
FERMENTATION OF CORN-MALT¹ MASHES AND THE
RETENTION OF ALPHA-AMYLASE

Papain added to yeast medium	Inoculum	Weight loss in grams				Final pH	Yield of alcohol Proof gal./bu.	Alpha-amylase activity units/ml. ²			
		19 hr.	27 hr.	43 hr.	69 hr.			0 hr.	19 hr.	43 hr.	69 hr.
Mg./100 ml.	Percent										
None	2	6.8	10.7	15.0	16.4	4.1	4.88	0.7	0.5	0 ³	0 ³
10	2	11.8	13.4	16.5	16.6	4.5	5.05	0.7	0.5	0.2	0.1
None	10	8.7	12.6	14.9	16.3	4.3	4.97				
10	10	13.4	14.4	15.5	16.7	4.5	5.03				

¹ All mashes were converted with 8% malt.

² Units are defined by Olson, Evans, and Dickson (6).

³ Ten ml. of mash failed to dextrinize in 30 minutes at 20°C.

Inoculum composed of 2% of papain-treated yeast gave a more rapid fermentation rate than either 2 or 10% of untreated yeast. The final pH values were again found to be higher in the mashes inoculated with yeast cultivated in the presence of papain. Likewise the latter, in contrast to the slightly more acid control mashes contained demonstrable alpha-amylase activity throughout the fermentation period.

Discussion

The effectiveness of certain proteolytic enzymes as a partial replacement for barley malt in grain alcohol fermentations has been demonstrated in the experiments described above. When introduced at the conversion step along with suboptimal levels of malt (5% of the grain), proteolytic enzymes accelerate the rate of fermentation and produce yields of alcohol about equivalent to those obtained with 8 to 10% malt. It was also observed that the pH during fermentation

remained slightly higher in papain-treated mashes than in mashes which received only malt.

Explanation for these effects was sought by examining the influence of proteolytic enzymes upon the rates of saccharification and fermentation. That the action of papain is confined to accelerating the rate of fermentation is shown in Fig. 1. From analyses of the free amino nitrogen contents of fermenting and non-fermenting mashes it was indicated that mashes which were converted with suboptimal levels of malt might fail to satisfy the requirements of the yeast for available nitrogen. This view was confirmed by the addition of casein hydrolyzate to similarly saccharified mashes. From the data in Tables III and IV, it is indicated that malt, even at a 10% level, fails to provide the amount of suitable nitrogen needed for a near maximum rate of fermentation. Therefore, the first effect noted upon reducing malt is a limitation of the yeast's ability to ferment available maltose, rather than inadequate saccharification as might be expected. Conversely, when metabolizable nitrogen is supplied, the activity of the yeast is markedly accelerated and bacterial activity as measured by the formation of acid seemingly is suppressed. The maintenance of a less acid reaction in turn improves the stability of malt alpha-amylase, thus favoring complete conversion.

While both casein hydrolyzate and urea are markedly active in stimulating fermentation, casein hydrolyzate is superior when the two supplements are compared on an equivalent nitrogen basis. As has been suggested by earlier workers (7, 9) this quantitative difference is probably attributable to the fact that certain amino acids satisfy specific nutritional requirements of the yeast. That these components may be absorbed in substantial amounts by culturing the seed yeast in amino acid-rich media is suggested in the present experiments. It should also be pointed out that protein hydrolyzates would vary in effectiveness according to their content of these constituents.

The lowering of alcohol yields by high levels of nitrogen supplement also is more pronounced with urea than with casein hydrolyzate. In view of the facility with which yeast cells assimilate urea and ammonia nitrogen, it is reasonable to assume that urea nitrogen is largely fixed in pyruvic and other keto acids which are formed during fermentation thus shunting these intermediates from fermentative to assimilative mechanisms. Brockmann and Stier (2) have recently expressed a concordant view and have hypothesized further that such assimilative reactions are essential to an accelerated rate of fermentation since they dissipate high energy phosphate bonds accumulating during the conversion of carbohydrates to alcohol.

While the substitution of malt was accomplished in these experiments by the addition of papain, ficin, and the enzymes from *B. subtilis* cultured in stillage, a similar result might be obtained by the use of malts with greater proteolytic activity or by the supplementation of seed yeast media with available nitrogen.

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FERMENTATION CABINET FOR 100-GRAM DOUGHS¹

H. E. RASMUSSEN and J. ANSEL ANDERSON

ABSTRACT

A square cabinet (38×54×32 inches) encloses an upright cylinder in which doughs are held on five circular shelves attached to a common shaft and rotated at 3 r.p.m. by a friction drive mounted below the cabinet. Air is conditioned beneath the cylinder and is circulated, by a centrifugal blower, up through the perforated shelves and down between cylinder and outer walls. A dry-bulb thermoregulator controls temperature at $86^{\circ}\pm 0.08^{\circ}\text{F}$. by operating heating lamps installed between the cylinder and outer walls. A wet-bulb thermoregulator, in front of the blower inlet, controls humidity at $90\pm 1\%$ by operating a Bahnsen-type humidifier inserted through the bottom of the cabinet. Rubber mounts and external location for all motors minimize vibration and heat input. The cabinet is made almost entirely of aluminum and is rustproof throughout.

A fermentation and proofing cabinet, with precise control of temperature and humidity, has been constructed to accommodate 100-g. doughs for a continuous baking schedule in which doughs are mixed at 5-minute intervals and proofed to time. Although at least two cabinets for test baking have previously been described (1, 2), and several types can be obtained commercially on the continent, certain features of the new cabinet seem to merit description because of their wide application in designing laboratory cabinets for various purposes. Accordingly, a general description of the cabinet, detailed descriptions of principal parts, and information on operation are given in the following three sections.

General Description

Fig. 1 shows a photograph of the equipment. The aluminum cabinet (38×54×32 inches) encloses an upright cylinder in which dough containers are held on five circular shelves attached to a common shaft. Air is conditioned below the cylinder and circulated up through the perforated shelves and down between cylinder and outer walls. Horizontal rotation of shelves and vertical circulation of air insure uniform conditions for all doughs. The cabinet stands on a welded angle-iron frame, 21 inches high, enclosed with plywood panels and doors.

Fig. 2 illustrates the design with an exploded view of the cabinet. The shelf assembly, which is shown partially withdrawn, drops into the surrounding cylinder, to which the bearing spider is then bolted. The cover fits over the casing and is secured by screws.

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At each of the front corners of the cabinet a long inspection door gives access to the air-conditioning chamber and to a cavity which houses sockets and wiring for heating bulbs. A cutaway portion of the insulated double wall shows the position of the bulbs.

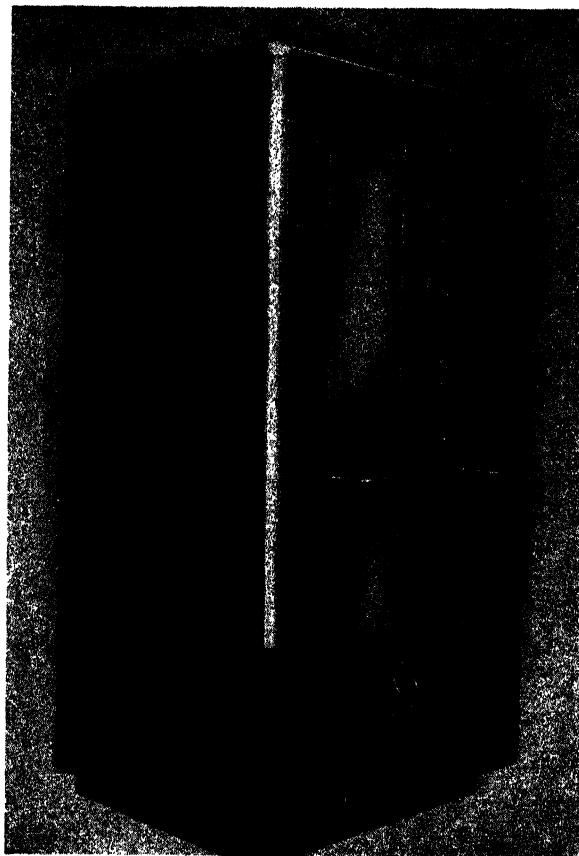


Fig. 1. Photograph of fermentation and proofing cabinet.

The horizontal partition, with four triangular holes for downward air flow and two circular holes for upward flow, fits within the casing against the bottom of the cylinder and is secured by rivets. When the base plate and blower compartment are riveted in place, an enclosed duct for air distribution and a re-entrant cavity for the blower are formed. The wet-bulb thermoregulator and its reservoir (on a small stand which is not shown) are placed at the left against the blower compartment with the wick in the direct draft; the dry-bulb thermoregulator is attached to the compartment on the right.

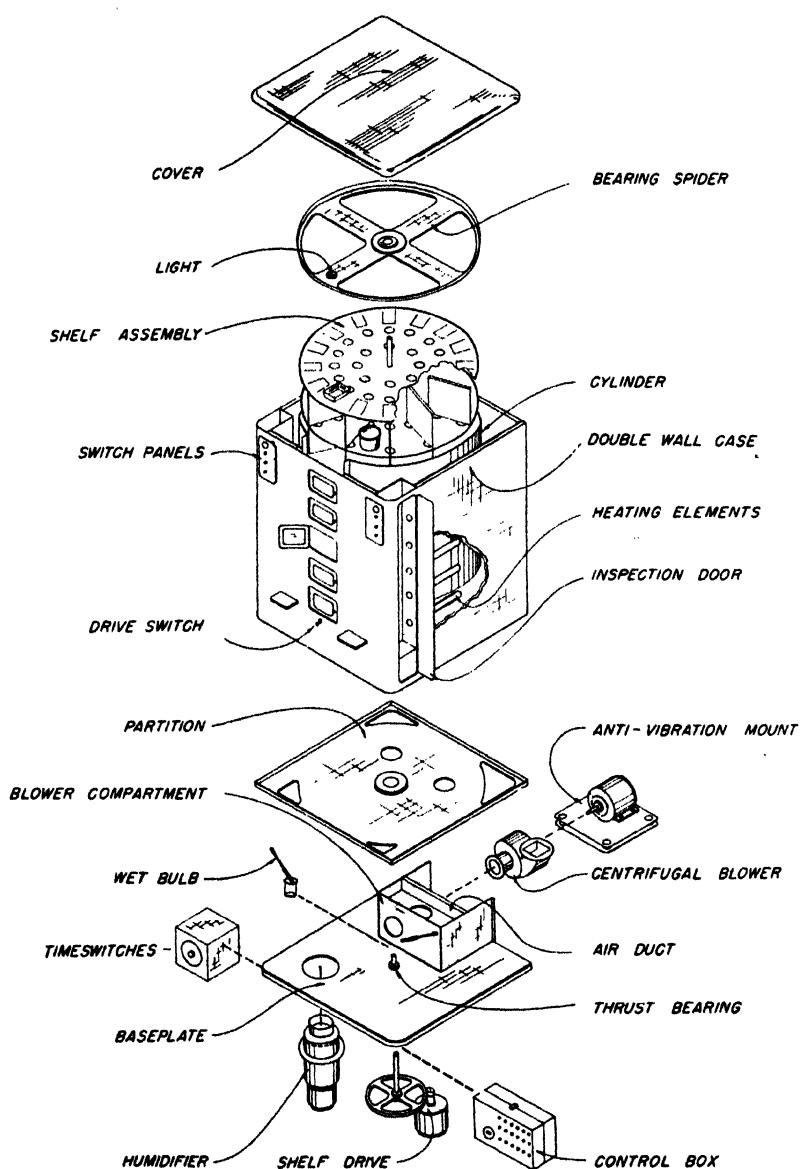


Fig. 2. Drawing of exploded view of cabinet.

The upper part of the humidifier is inserted into the conditioning chamber through a hole in the base plate. The lower part of the humidifier, a box containing time-switches, the drive for the shelves, and a control box containing electrical accessories are housed within an enclosed stand beneath the cabinet (Fig. 1).

Dry- and wet-bulb temperatures of 86° and 83.5°F., giving a relative humidity of 90%, are maintained in the cabinet. Variations of these conditions with time are minimized by using mercury-in-glass thermoregulators sensitive to $\pm 0.05^{\circ}\text{F}.$, by placing the wet bulb directly in front of the blower inlet, and by adjusting rates of heat and vapor inputs to give short control cycles with little overrun. Variations in space are minimized by rapid vertical circulation of the air assisted by the mixing action of the rotating shelf assembly. Moreover, small doors and the shielding effect of shelf partitions minimize variations in cabinet conditions during insertion or removal of doughs.

Four general features of the design deserve mention. Firstly, as the temperature of the baking laboratory is maintained at 78°F. and rises higher for a few weeks during the summer, precise control of the cabinet at only a few degrees above room temperature requires that heat input be reduced by external mounting of drive, blower, and humidifier motors, and of the heated humidifier reservoir. Secondly, vibrations that might affect doughs are prevented by ball bearings for the shelf assembly, a drive that starts and stops smoothly, and anti-vibration mounts for all motors. Thirdly, all working parts of the equipment are readily accessible for servicing or eventual replacement. And fourthly, as the baker stands in the same place to insert and remove doughs on all shelves, the cabinet can be conveniently placed to minimize steps required during the baking schedule. In short, though the cabinet is relatively complex, it has been designed to provide convenient operation and long, trouble-free service.

Detailed Descriptions of Principal Parts

The following subsections describe the main details of construction of the cabinet and of the various auxiliary parts: drive, humidifier, blower, controls, wiring, and time switches. The complete unit was designed in this laboratory. Sheet metal work was done by a Winnipeg firm, but all other construction and all installations were undertaken in the laboratory.

Construction. Except for the base plate, the cabinet is constructed entirely of $\frac{1}{16}$ -inch aluminum plate (3S4H) with all joints riveted. The base plate consists of a $\frac{1}{4}$ -inch aluminum plate supported on an angle-iron frame with channel-iron cross braces. A vertical thrust bearing at the center of the base plate carries the full weight of the shelf assem-

bly. The shaft, which is $\frac{1}{4}$ -inch stainless steel, is also supported above and below the cylinder by horizontal thrust bearings. Sealed ball bearings, glass-wool insulation, and rustproof materials for the cabinet and all auxiliary parts minimize the injurious effects of high humidity.

Drive. Rotation at about 3 r.p.m. was required for the shelves. An ordinary motor and reducing gears would start the shelf with an undesirable jerk and would require a relatively complex clutch mechanism for bringing selected dough containers into position for removal. These difficulties were overcome by a friction drive and a disc induction motor (G.E. type AZ-133A) such as is used for turning record tables in broadcasting stations. The motor has a built-in governor which per-

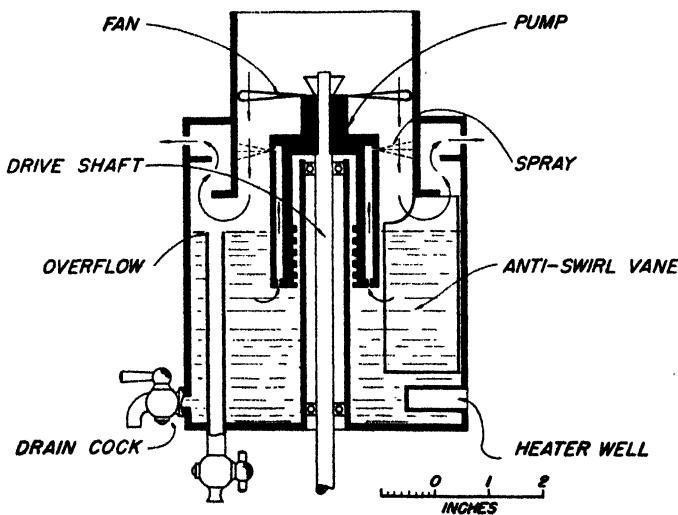


Fig. 3. Cross-section drawing showing the design of the humidifier.

mits the speed to be adjusted to 65 r.p.m. A No. 6 stopper on the motor shaft rotates the shelf assembly by driving against the rim of a 12-inch pulley attached to the shelf shaft below the bottom of the cabinet. Two Lord mounts serve as hingelike supports for the motor and allow its weight to hold the stopper against the pulley (Figs. 2 and 4). This drive permits the baker to swing the shelves by hand, either forward or backward, to bring any crock into position for removal within a second or two. In addition, the motor is silent, runs with minimum vibration, and starts and stops the shelf slowly and smoothly.

Humidifier. Fig. 3 shows a drawing of the humidifier. It is of the Bahnsen type but with the design modified so that the motor is mounted beneath rather than above the unit. This change involved construction of a reservoir with a central bearing housing extending

above the water, and of a modified pump running on the outside of this housing.

Rotation causes four slanted openings in the bottom of the pump to drive water up between the sleeves and out of four pin-holes near the top to form a horizontal curtain of fine spray in the chimney. The fan at the top of the chimney blows air down through the spray, over the surface of the water, and out through openings around the top of the reservoir. Water is prevented from being thrown up between the pump and bearing housing by a reverse thread cut on the inside of the pump at the bottom.

Efficient humidification requires that the water be maintained 10° to 15°F. above the temperature of the cabinet. A well is therefore provided to accommodate a constant heater consisting of a standard 20-w. tip for a soldering iron. The unit is self-balancing; if the operating time of the humidifier increases, the greater heat loss through evaporation causes the temperature of the water to fall slightly, and vice versa; equilibrium is attained when heat loss through evaporation and radiation equals heat input. The reservoir is filled by opening the stopcock on the overflow pipe and pouring water down the chimney until a few drops overflow.

Other details of construction are shown in the drawing: baffles to remove entrained water from the air stream; antiswirl vanes; overflow pipe; and drainage cock. The motor (Bodine, type NSE-11R, 1/40 h. p.) is mounted with Lord mounts on three columns on the bottom of the reservoir. By lowering the voltage to 85 with a transformer, the speed of the motor is reduced to 3,500 r.p.m., so that the unit will run almost silently and with negligible vibration.

Blower. The centrifugal blower consists of a standard rotor, 3 inches wide by 5 inches diameter, driven at 1,725 r.p.m. by a $\frac{1}{8}$ -h.p. motor, and delivering approximately 40 c.f.m. In order to prevent transmission of vibration by avoiding direct contact between blower and cabinet, a special rotor housing was constructed (brass, nickel plated) to provide a collar and flange at the inlet and a flanged outlet (see Fig. 2). When the unit is in place, inlet and outlet flanges are centered within larger holes in the blower compartment, and the annular spaces are closed by rings of $\frac{1}{4}$ -inch rubber sealed against flanges and compartment by suitable clamping rings. The motor, with rotor housing attached to it, is supported on four pairs of Lord mounts which effectively damp both vertical and transverse vibrations. Fig. 4, a photograph of the back of the cabinet, shows the assembly. The transformer on the left decreases the running temperature of the motor by reducing voltage to 90, and the small fan on the back of the motor keeps the temperature down within the cavity.

Controls. The thermoregulators (8-inch, Aminco No. 4-201) actuate battery-operated relays (Kurman, No. 190036). Three 40-w. showcase lamps, 12 inches long, on each side of the cabinet are controlled by the dry-bulb thermoregulator. Two lamps in series are connected in parallel with the third, so that 60 w. are provided on each side. Four additional 40-w. bulbs provide stand-by service for more rapid heating of the cabinet.

The wet-bulb thermoregulator controls the humidifier. When the wet-bulb temperature falls and breaks the contact in the thermoregu-

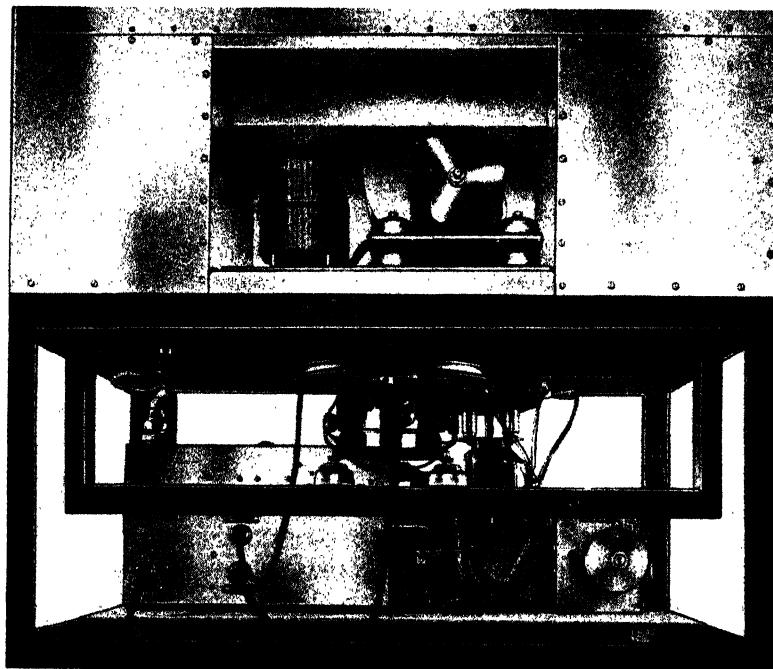


Fig. 4. Rear view of cabinet with panels removed from stand.

lator, the humidifier motor starts and runs until contact is re-established. As the cabinet is not completely airtight, humidity tends to fall when the humidifier stops through interchange of air with the room. Sensitive operation is insured by locating the wet bulb immediately in front of the blower inlet. The thermoregulator and its reservoir (100-ml. beaker) are mounted on a small stand which can be readily removed through the left-hand inspection door for servicing; this involves rinsing the sock and filling the reservoir.

Wiring. As the cabinet operates at high humidity, most of the wiring and accessory electrical equipment is housed in a separate con-

trol box beneath the cabinet. This is shown at the left in Fig. 4. The box contains the thermoregulator relays and their batteries, fuses for each circuit, and certain auxiliary switches. Leads from within the cabinet and from the time switches are brought into the box by Cannon connectors; and individual outlets are provided for plugging in the main lead supplying 110-volt current, the three motors, and the humidifier heater.

The principal switches are mounted on the face of the cabinet, the drive switch below the doors, and the others on panels near the top corners (Fig. 1). In addition to the master switch, three others are provided to control the blower and heating units, the humidifier, and the cabinet light. Two switches for auxiliary heaters are mounted on the control box.

Time Switches. Intermittent operation of the cabinet is advisable to dry it out daily and to reduce wear on working parts. In order that the cabinet may be ready for immediate use in the morning, time switches have been installed to turn it on before the baker arrives. The assembly is housed in the metal box shown on the right in Fig. 4. A microswitch, operated by a 24-hour cam, closes circuits for the blower, controlled heaters, and humidifier heater at 4:30 a.m. By 8:00 a.m., the cabinet temperature is controlled at 86°F. and the humidifier water has reached 105°F.; the humidifier is then turned on by a second cam and microswitch. Humidity rises to 90% in about 10 minutes, during which time the water temperature drops to the operating level of 98° to 100°F. These two switches also turn the cabinet off at 4:30 p.m. A third microswitch, with a 7-day cam, prevents the cabinet from operating on Saturdays and Sundays by opening the line to the other two microswitches. A submaster switch on the panel, marked "operation," is used with the time switches; it opens all circuits except that for the clock motor.

Operation

Although the cabinet is relatively complex, its operation is simple. During the week, the baker has only to move the "operation" switch when he decides whether or not he will need the cabinet next day. In addition, he must service the wet-bulb thermoregulator and fill the humidifier, which takes about 3 minutes.

The dry-bulb and wet-bulb thermoregulators are sensitive to less than $\pm 0.05^{\circ}\text{F}.$, are accurately set at 86° and $83.5^{\circ}\text{F}.$, and maintain their settings indefinitely. Because of slight overshooting of the control level when heat is added, the variation in dry-bulb temperature is $\pm 0.08^{\circ}\text{F}.$ Equipment was not available for measuring the corresponding variation in the wet-bulb temperature, but this is probably of the same order.

As the thermoregulators operate independently, one may be at its maximum while the other is at its minimum and vice versa; accordingly, the spread between dry- and wet-bulb temperatures probably varies between 2.34° and 2.66°F., which is equivalent to a variation of less than $\pm 1\%$ in relative humidity. But operational cycles are short; for heaters, 3 minutes on and 11 minutes off; and for the humidifier, 0.5 minute on and 5 minutes off. Malloch (3) has demonstrated that doughs cannot pick up such rapid variations in temperature, and observation shows that this is true of humidity variations also. Thus, for all practical purposes, the cabinet may be said to operate at absolutely uniform temperature and relative humidity in time.

Since the doughs rotate horizontally, effective variations of conditions in space are restricted to those that occur between shelves. Observations made with precise thermometers show that the rapid vertical circulation of air reduces the difference in temperature between top and bottom shelves to about 0.1°F. This is negligible.

Data on the reproducibility of loaf volumes have been compared for the new cabinet and its predecessor. Direct comparisons could not be made because expediency required that the old cabinet be retired to make room for the new one. Unfortunately, indirect comparisons are less satisfactory because reproducibility varies with both handling properties and loaf volume, though the effect of the latter can be partially offset by calculating coefficients of variation. The last six substantial investigations made with the old cabinet gave coefficients, arranged in increasing rather than in chronological order, of 1.4, 1.5, 1.5, 1.6, 1.8, and 2.1%; and the two large investigations made with the new cabinet gave coefficients of 1.2 and 1.4%. Accordingly, there is some indication, though no convincing proof as yet, that reproducibility has improved. Irrespective of whether this hypothesis is substantiated, the convenient operation, easy servicing, and durability of the new cabinet suggest that the cost of engineering and constructing it will prove to be a sound investment.

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RELATIONSHIP OF DIASTATIC ACTIVITY TO FLOUR STRENGTH¹

C. C. WALDEN² and R. K. LARMOUR³

ABSTRACT

Two types of gassing rate curves, due to the fermentation of maltose produced by flour diastatic action, were observed.

Autolytic maltose value and protein content of flours are related inversely.

With baking formulas capable of maintaining adequate gassing rates to oven time, differentiation between flours of varying strength occurs mainly in the oven, rather than during the proof period. The practice of proofing to constant height should not be followed in evaluating total flour strength.

The autolytic determination of the diastatic enzymes of flour devised by Rumsey (10) and further refined by Malloch (8) and Blish and Sandstedt (3) provides the method now widely used to determine the maltose-producing ability of flour. The maltose formed may be estimated by any conventional physical or chemical method applicable to sugars.

The manometric method (5), which parallels dough conditions, measures the pressure developed by the fermentation of the sugars produced in the autolytic system. The degree of relationship between this method and the autolytic maltose value is high, although the latter does not include the preformed sucrose in the flour. Gas production may be measured volumetrically (2), this method permitting a ready evaluation of gassing rates.

The current concept of the autolytic production of flour maltose is that of an activity of excess beta amylase under limiting substrate conditions. Following the observation of Alsberg and Griffing (1) on the effect of fine grinding in increasing maltose production, various workers have studied the effect of starch injury on autolytic diastatic activity. Dadswell and Wragge (6) concluded that diastatic activity is correlated with starch injury resulting from milling, and Jones (7) determined that the maltose figure is dependent on the content of damaged starch granules. In the absence of diastatic supplements, the maltose figure is a measure not of the beta amylase but of the degree of mechanical injury occurring to the starch granules during the milling process.

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Munz and Bailey (9) observed that the higher the maltose value of a given flour, the longer was its effective gassing period, and that additions of sucrose eliminated these differences between flours. Walden and Larmour (13) have shown that a high yeast, high sugar, high salt formula is necessary if the baking data are to reflect strength of the flour rather than the limitations of formula. This paper deals with further work on the relationship between the maltose-producing ability of varying flours and their behavior with the above type of formula.

Materials and Methods

The series of flours used consisted of 96 flours, 16 each from the following bread wheat varieties—Marquis, Thatcher, Apex, Renown, Regent, and Rival. The protein variation for the entire series was from 7.9% to 18.6% (13.5% moisture basis) with a variation of the same order within each variety. As the inclusion of all the data obtained with these six series would encumber this report unduly, only a few typical examples will be presented. The complete original data are on record (Walden, 12).

Natural gassing rate curves are those of 3% yeast, flour, water doughs. Baking data and gas retention measurements were obtained with the following formula: 3% yeast, 1.75% salt, 5% sugar, 4% nonfat milk solids, 3% shortening, 0.1% ammonium dihydrogen phosphate, 0.3% 20° Lintner malt, 0.001% potassium bromate. Pup loaves from 100 g. doughs were mixed, fermented, proofed to constant time, and baked according to the A.A.C.C. procedure. Gassing rates and gas retention were measured as outlined by Walden and Larmour (13). Maltose values were determined by the method of Blish and Sandstedt (3).

Results

Baking Data. Using the above formula, which provides an adequate rate of gassing through the fermentation and pan-proof periods and into the oven period (13), baking tests were made on the above series of flours. The statistical data are summarized in Table I. The correlations between protein content and loaf volume indicate that where gassing rates are adequate, there are few remaining variables influencing the relationship of loaf volume to gluten quantity. Variation in gluten quality within this series appears to be small.

Natural Gassing Power. Thirty-two flours of the original series, comprising all the Marquis and Thatcher samples, predominant Western Canadian varieties, were examined for natural gassing power. Fig. 1 reproduces the gassing rate curves of a Marquis subseries and illustrates the various phenomena encountered in all the flours studied.

TABLE I
STATISTICAL CONSTANTS FOR LOAF VOLUME AND PROTEIN CONTENT

	Entire series	Apex	Marquis	Rival	Renown	Regent	Thatcher
Mean protein of flour \bar{x}	13.88	13.96	13.46	13.58	14.05	14.14	14.09
Mean loaf volume \bar{y}	923.4	905.2	908.5	901.3	930.1	935.3	959.8
Correlation coefficient r_{xy}	+0.971	+0.991	+0.979	+0.982	+0.988	+0.994	+0.963
Regression coefficient b_{xy}	+46.27	+37.08	+40.88	+52.64	+42.54	+46.04	+54.34
1% point	0.254				0.623		

All eight flours exhibit double maxima gassing curves, with a small variation in the first maximum in respect to height and time, and a pronounced differentiation in the second maximum. Walden and Larmour (13) concluded that the first maximum can be explained by

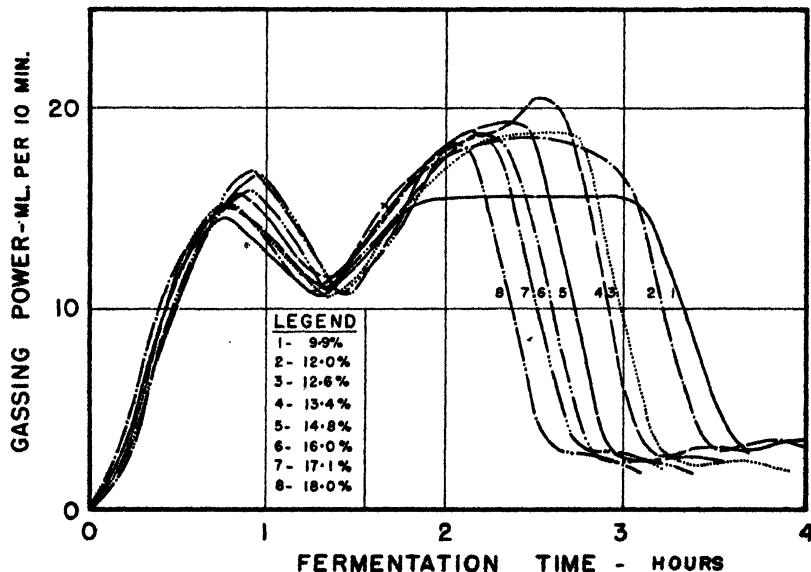


Fig. 1. Gassing rates of composite Marquis wheat flour doughs fermenting with 3% yeast only. Protein contents shown in legend.

the assumption that the initial fermentation uses the sucrose present in the flour, and when this becomes a limiting factor the rate falls off until the fermentation of maltose is initiated, whereupon the rate of fermentation accelerates again to produce the second maximum. The height and duration of the second maximum is directly related to the maltose-producing capacity of the flour. It is interesting to note in Fig. 1 that although there is some displacement of the first maximum, indicating that slight differences of sucrose content of the flours existed,

all the minima occurred at practically the same rate and time. Thus when the second acceleration of fermentation occurred, all the flours started on practically an equal basis, and the real differentiation in their gassing powers is evident in the latter part of the curves.

The two types of second maxima are indicative of the variations observed in all the flours studied. Above 10% protein the second maxima reach a sharp peak and then decline in order of descending protein content. At levels of 10% protein or lower, a flat extended maximum, as illustrated by curve 1, Fig. 1, was obtained. This plateau-type curve does not reach the maximum value shown by higher protein flours, but extends over an appreciably longer period. Without added baking supplements only the lower-protein flours could make any appreciable contribution to gassing during the pan-proof period (between 3rd and 4th hours according to A.A.C.C. procedure).

The data presented in Table I show that with a proper formula which will maintain the gassing rate, there is a very high correlation between protein and strength as indicated by loaf volume. It is obvious from Fig. 1 that unless adequate sugar, or diastatic supplement, or both are provided in the formula, the high-protein flours will be penalized unduly and that the low-protein flours will appear relatively much stronger than they really are.

While a lack of ability to maintain an adequate gassing rate through the fermentation period might be deemed a quality factor in a finished commercial flour, it ought not to be so regarded in estimating inherent strength of wheats because it can be readily changed either by additions to the flour itself or to the baking formula. In brief, gassing power is a function of the baking formula rather than of the quality of the wheat or flour.

Correlation of Maltose Figure and Protein Content. The entire series of 96 flours was analyzed for autolytic maltose value and the data, shown graphically in Fig. 2, reveal clearly the inverse relationship with protein content. The statistical constants for maltose value and protein content are as follows:

Mean protein of flour \bar{x}	13.88
Mean maltose value \bar{y}	188.19
Correlation coefficient r_{xy}	-0.724
Regression coefficient b_{yx}	-9.074
1% point	0.254

The correlation coefficient, -0.724, although not high, is significant.

This explains in part the extended gassing power of the lower-protein flours: they produce more maltose. But it fails to explain why

the rate of gassing, although more extended, is never as high as in the high-protein flours.

Gas Retention. Loaf volume is the sum of the dough volume immediately after panning (virtually a constant) and the volume of gas retained during the pan-proof and oven periods. Where gas production has been eliminated as a variable, the remaining variable is the ability of the dough to retain the gas. Having developed such condi-

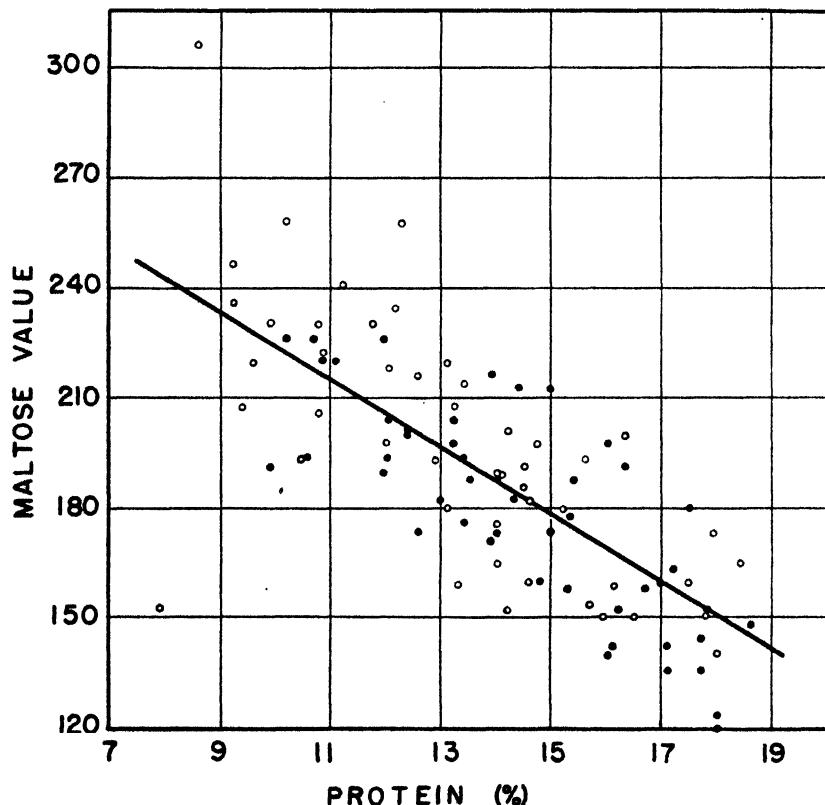


Fig. 2. Scatter diagram showing relation between maltose value and protein content.

tions, it is possible to evaluate the role of the pan-proof and oven period in gas retention.

Proof height is frequently used to indicate differences in proofing ability. A more accurate, but not routine, measurement of this property is a volumetric measurement of dough volume increases. Gas retention measurements on the high yeast, high sugar, high salt formula, given graphically in Fig. 3, are for those flours whose natural gassing rate curves are shown in Fig. 1. The left-hand group of curves

gives dough volume increases from time of mixing to the beginning of the pan-proof period. At the beginning of the pan-proof period, measurements were continued on fresh dough aliquots which had been fermented and punched since mixing time according to the A.A.C.C.

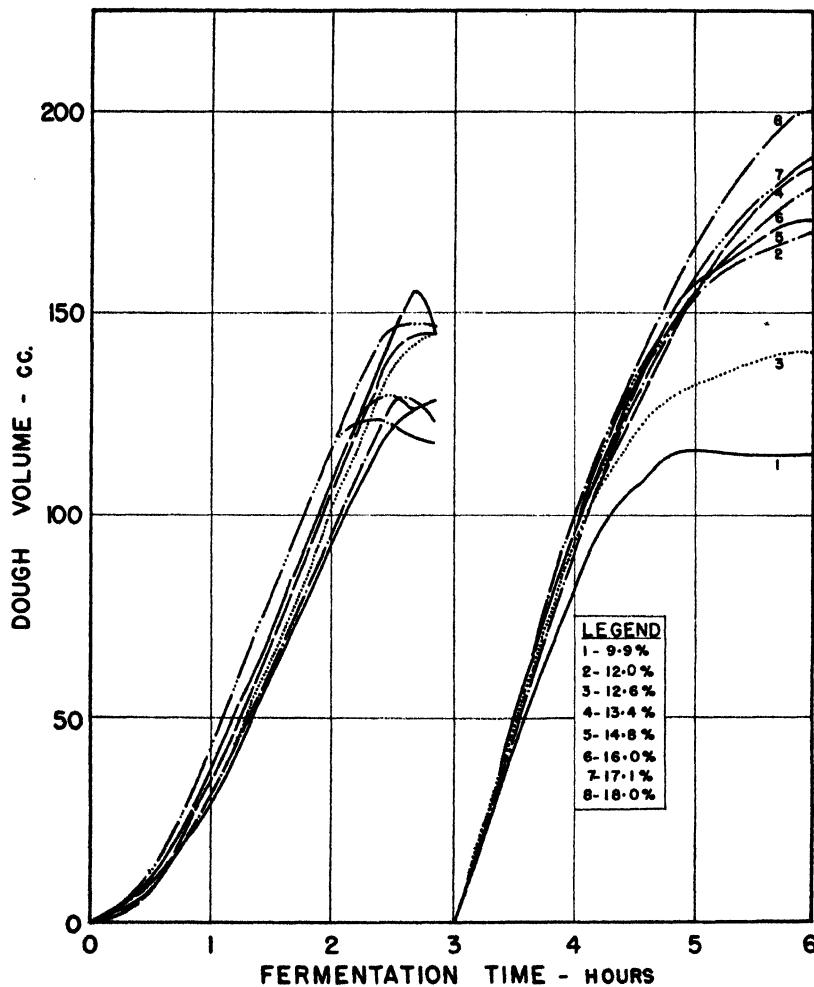


Fig. 3. Dough volume changes of composite Marquis wheat flour doughs fermenting on baking formula.

Left-hand group of curves—from time of mixing the dough.

Right-hand group of curves—from beginning of pan-proof period.

procedure. Dough volume increases for these doughs from 3 hours to 3 hours and 55 minutes correspond to pan-proof increases.

Dough volume increases to the end of the pan-proof period on the basis of 100 g. flour are shown in column 2 of Table II. These values

are smaller than the actual dough volumes at oven time by the volume of the dough after panning. Correspondingly the oven springs, obtained by subtracting the dough volume increases from the loaf volumes, are too great by this quantity.

TABLE II

DOUGH VOLUME (PAN PROOF) INCREASES, LOAF VOLUMES, AND OVEN SPRINGS FOR MARQUIS SERIES

Protein content	Dough volume increase (basis 100 g. flour)	Loaf volume	Oven spring
%	cc.	cc.	cc.
9.9	300	776	476
12.0	332	846	514
12.6	342	832	490
13.4	338	894	556
14.8	350	961	611
16.0	346	1019	673
17.1	352	1055	703
18.0	362	1114	752

The range in dough volumes corresponding to a loaf volume variation of 338 cc. is only 62 cc. Even assuming that the gas retained in the pan-proof period expands thermally to oven temperature within the dough before crust formation, this range is increased only to 104 cc. This is a theoretical limit only, such conditions never being reached under actual baking conditions. The oven spring parallels loaf volume, and differentiation between flours on this basis is much greater than obtained with dough volumes. Similar results were obtained on 24 additional flours (12).

Differentiation between flours of varying strength, baked by a rich formula, occurs mainly in the oven, and the dough volume, which in actuality is a more accurate measure of proof height, is of minor significance.

Discussion

The longer period of effective gassing by low protein flours is due to their greater maltose-producing ability. With the current concept of the autolytic maltose figure, this must be attributed to a greater total content of mechanically damaged starch. From their lower protein content it follows that these flours possess a higher total starch content, although milling damage is normally associated with kernel vitreousness or high protein content.

Walden and Larmour (13) have shown that with a high yeast, high salt, high sugar formula the fermentation of autolytic flour maltose is retarded to the point where it may be of little significance under the A.A.C.C. baking procedure. The foregoing evidence shows that if

maltose fermentation is a contributing factor before crust formation, it is not a critical one: gas production has been eliminated as a variable and the doughs, having adequate gas production during the pan-proof and oven periods, have retained the gas to their maximum ability to do so. As a result, baking data show a high correlation between loaf volume and protein content. Under such conditions, the authors believe that the resulting loaf volume reflects fundamental flour strength.

The laboratory practice of proofing to constant height was recommended to equalize differences in "Factor M" content between flours (11). "Factor M" is a biocatalytic activator, specific to maltose fermentation, the existence of which was postulated by Blish and Sandstedt (4). Since it has been shown that neither maltose production nor maltose fermentation in a dough have any critical influence on baking strength with rich formulas, the necessity of proofing to constant height is obviated. Further studies on this type of formula have indicated that the major differentiation between flours occurs not during the proof period, but in the oven. There is a tendency for differentiation of flour strength during the pan-proof period, but it is not reliable. Nevertheless the authors maintain that the minor contribution pan-proofing makes to loaf volumes under adequate gassing conditions is due to differences in flour strength and any attempt made to equalize proofing rates will mask these differences.

In commercial baking with lean formulas, proofing to constant height may be of value in maintaining a uniform product. Experimental baking, designed to imitate commercial practice, may also find proofing to height of some value.

But in other phases of the milling and baking industries, where the volume-producing potentialities of a flour are of utmost importance, proofing to height has no place in the baking procedure.

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MICROSCOPICAL OBSERVATIONS AND PROCEDURES USED IN A STUDY OF INDUSTRIAL CORN STEEPING¹

JOHN A. WAGONER²

ABSTRACT

A microscopical study was made of the protein matrix and the cellulose walls of the corn endosperm while the grain proceeded through the usual industrial wet milling steeping process. Cross sections of the steeped grain were made with a freezing microtome. Starch was cleared with chloral hydrate; protein and cellulose were differentially stained with thionine. The method gave rapid, reproducible results in which the occurrence of small changes could be distinguished microscopically.

The lactic acid phase of the steeping process softened the corn by the absorption of water. In the later portion of the process the sulfurous acid concentration was high and the protein matrix was disrupted, leaving the protein massed against the cellulose cell walls. Additional steeping loosened this protein from the cellulose walls.

Corn stored a year was steeped along with some freshly harvested grain, and it was shown that the protein network of the new corn was disrupted more rapidly. This was not caused by a difference in the moisture content, since both samples attained the same moisture content early in the steeping.

In the wet milling process for the manufacture of corn starch, there is considerable plant readjustment in the fall of the year when the new corn is started through the process. It has been observed over a period of years that if sound new corn is steeped for a shorter length of time and the concentration of the sulfurous acid reduced, it could usually be processed in about the same manner as the old. However, since this is not always the case, a microscopical study was started to ascertain the structure of a well-steeped kernel of corn and the differences between old and new corn during the processing.

Kerr (2) describes a number of methods of commercial processing of corn. Practically all of the wet milling industries use the counter

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current method, or a modification thereof, in which a sulfurous acid solution (about 0.25%) is added to the almost fully steeped corn. This solution is then pumped out of the bottom of that steeping tank to the tank in which corn has been steeping for a slightly shorter length of time. This procedure is continued throughout a battery of steeps so that the solution placed on the dry corn has been transferred through the line of steeping tanks. As the solution is circulated, the sulfurous acid is absorbed by the corn so that its acidity is reduced from 0.25% to 0.02%. Thus the most completely steeped corn is processed in essentially a sulfurous acid steep. Most of the sulfurous acid has been absorbed during the first 10-12 hours, leaving conditions which favor the production of lactic acid by fermentation. Thus as the steeping is continued, the water pumped on the corn which first enters the process has a high lactic acid, and a low sulfurous acid, content. During the whole cycle the procedure has been regulated so that these two acids counterbalance to keep the system within a pH range of 3.5 to 4.5. Most of the industries steep their corn from 36 to 50 hours at temperatures from 115° to 130°F.

Studies of microscopic examinations of corn during steeping have been published by Cox, MacMasters, and Hilbert (1). However, their steepings were for shorter periods of time, and the corn was steeped immediately in the most concentrated sulfurous acid solution without the lactic acid steep encountered in industrial practice. Thus, while it was decided to use essentially their methods of sectioning, the products investigated had been subjected to quite different treatment.

Materials and Methods

Corn Samples. Since very little was known about the actual progress of steeping within a kernel of corn, samples of corn were removed from the steeping tanks at all stages in the steeping cycle. Each sample of corn was sectioned, stained, and mounted within an hour after the sample was removed from the tanks. In this manner progressive changes were followed on corn which was considered to be processing normally. Later, when new-crop corn was received, a study of comparative steeping of old and new corn was made using as receptacle a wire basket divided in the center and containing about a pound of each. The charged basket was lowered into a steeping tank as soon as the tank had been filled, and at 4-hour intervals samples of about 25 g. of each were removed for study.

Moisture Analysis. The sample was rinsed immediately to remove the adhered solubles, and the surface moisture blotted. Its moisture content was determined by drying to constant weight in an oven maintained at 110°C.

Microscopic Examination. Several kernels of average shape and size were sectioned, using a Spencer sliding microtome. First a cross-sectional cut was made about halfway between the upper end of the scutellum and the crown; another was cut about 3 mm. closer to the tip. This center section was placed upon the object holder in the freezing chamber and mounted in a 10% solution of a water-soluble

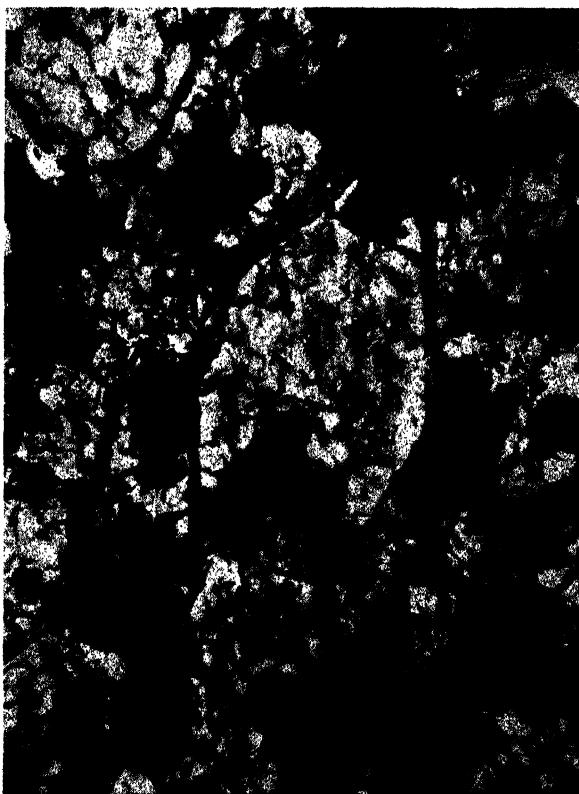


Fig. 1. Cross section of old corn steeped 12 hours. This section is from the middle of the horny endosperm, midway between the tip and the crown. The protein matrix is intact, and the cellulose walls are very strong. $\times 430$.

gum, such as gum arabic or talha. By using this gum solution, the sections could be obtained with very little tearing of the pericarp from the endosperm. The section was frozen by carbon dioxide gas, care being taken to encase the sample completely with the gum solution. Freezing was continued for about 3 minutes by which time solidification was complete. Sections 40μ in thickness were cut, removed from the blade with a moistened camel's hair brush, and placed in distilled water. As soon as several good sections were obtained, one of them

was placed on a microscope slide and a drop of 0.05 *N* iodine solution placed on it. The iodine was allowed to stand 3 or 4 minutes and washed off. Then several drops of 75% chloral hydrate were added to the section, and after 30 seconds or so the starch granules could be brushed out of the section with a fine-hair brush. With these samples the chloral hydrate proved more satisfactory than the use of sulfuric



Fig. 2. Cross section of old corn steeped 36 hours, the section taken from same area as Fig. 1. The protein matrix is disrupted far into the horny endosperm, but the cell walls are still strong. $\times 430$.

acid described by Cox, MacMasters, and Hilbert (1). The completely steeped sections were very fragile, and an extra second or so of treatment with the acid would destroy the section, but a variation in treatment time of 30 seconds with the chloral hydrate had no appreciable effect. When light to moderately steeped grains were sectioned, either method gave good results. The section was then washed, and a few drops of 0.25% thionine solution, adjusted to a purple color with ammonia, were placed on the section. After it was allowed to stand 10 minutes, this solution was rinsed off. The protein was stained

yellow, the cellulose blue to reddish, and the starch remaining in the section appeared black. This section can be mounted in water if the sections are to be photographed immediately, but a temporary mount of a solution of polyvinyl alcohol will make the slide usable for several days.

Results and Discussion

Fig. 1 shows a section taken from a matured corn grain with moderately low moisture content which had been steeped for 12 hours.

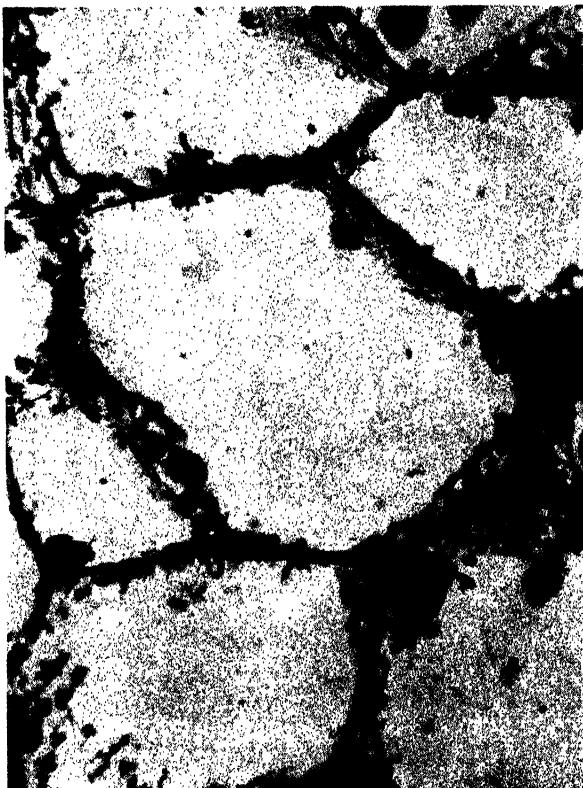


Fig. 3. Cross section of old corn steeped 50 hours, the section taken from the same position as Figs. 1 and 2. The protein matrix is disrupted out to the aleurone layer; the protein is folded against the cell walls; and the walls are quite fragile. $\times 430$.

Up to this time the corn was very hard and sections were difficult to make. The protein matrix is still intact, the starch is held tenaciously, and the cellulose walls are very firm. Only slight changes can be noticed in any of the sections until the corn has been steeped for about 24 hours. At this time the sulfurous acid content of the steepwater is beginning to rise quite rapidly, and the protein matrix in the floury

endosperm begins to break up, freeing the large starch granules. At 36 hours the protein network has dispersed through most of the horny endosperm as shown in Fig. 2. Continued steeping furthers this disorganization until the protein network has disappeared even around the small granules next to the aleurone layer. A well-steeped kernel of corn is illustrated in Fig. 3. This corn had been steeped for 50 hours, and it is noticed that there is no protein matrix, but the cellulose cell



Fig. 4. Cross section of new corn steeped 36 hours. The section was taken in the same position as Fig. 2. The protein matrix has been disrupted almost as much as in Fig. 3. The cell walls are very strong. $\times 430$.

walls are intact. Most of the protein network has dispersed in the acid and the remainder has folded back around the cellulose walls; also, the starch has been freed. The starch was easily separated from the protein giving a starch of moderately low nitrogen content.

When the new corn was steeped along with the old corn of lower moisture, no structural differences could be detected until after the 24th hour. After that time the protein matrix of the new corn broke

up much more rapidly, and by the 36th hour this corn was steeped as well as the old corn after 50 hours (Fig. 4). This was not caused by a difference in moisture content of the grain. Fig. 5 shows the moisture content of old and new corn during the steeping. The old corn had 14.0% moisture, while the new corn had 22.2%, but after 15

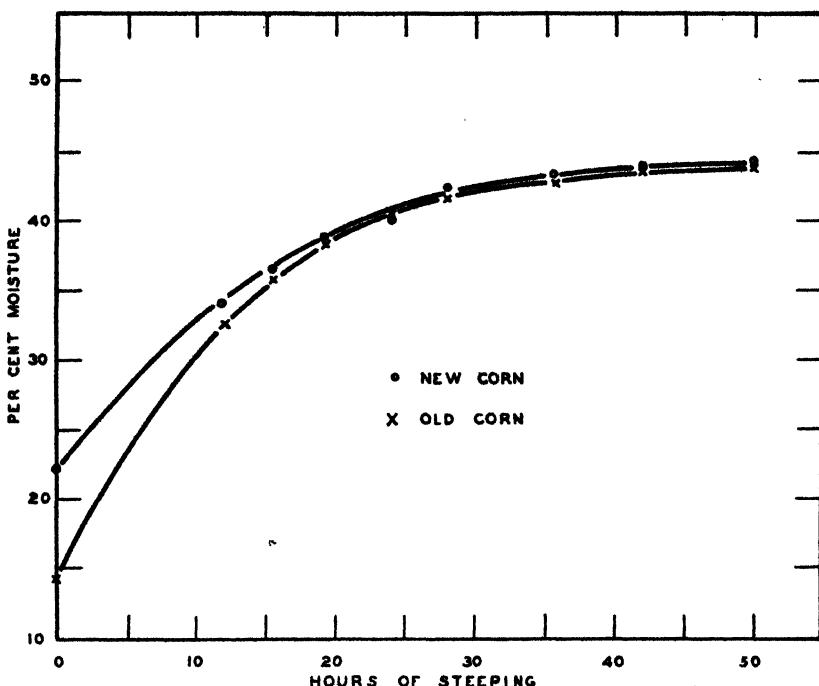


Fig. 5. Moisture absorption of old and new corn during steeping.

to 20 hours both were at relatively the same moisture content. During the rest of the steeping there was no appreciable difference between the two in moisture content. Thus moisture absorption is not a satisfactory criterion for steeping of corn. Continued steeping of the new corn loosens the protein from the cellulose cell walls leaving the cell sections almost free of protein (Fig. 6). This section was quite sturdy, and good ones were easy to obtain. The starch obtained from the new corn steeped for this length of time was higher in nitrogen than the average starch obtained.



Fig. 6. New corn steeped 50 hours. This section is from the same area as Figs. 1, 2, 3, and 4. The protein matrix has been completely disrupted and a considerable amount has been washed away from the cell walls. Section is 40μ thick. $\times 430$.

Acknowledgment

The author expresses his appreciation to Dr. B. M. Smitz of Kansas State College and Dr. M. M. MacMasters, and M. Cox of the Northern Regional Research Laboratories, for their suggestions upon this work.

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A SIMPLIFIED DETERMINATION OF THE COLD WATER ABSORPTIVE CAPACITY OF PREGELATINIZED FLOURS¹

LEO KLEE, LOUIS SAIR, and LLOYD A. HALL²

ABSTRACT

A simple, routine technique based on the volume of centrifuged flour-water pastes has been applied in the evaluation of cold water absorptive capacity of unprocessed and pregelatinized wheat and corn flours. This technique is more accurate than an older method based on the measurement of flow properties.

A simple, rapid method for evaluating flours used until recently in this laboratory involved a determination of the water absorption required to produce a paste of a given viscosity as judged empirically by its flow properties. This method, however, lacked precision because of the difficulty in judging the degree of flowability of the paste.

A swelling volume method has been used for the evaluation of starch pastes in research laboratories for several years. Brimhall and Hixon (1) have shown that there is a close relationship between the volume of swollen granules of corn starch paste and relative viscosity. Accordingly, in an effort to improve on the rather crude pasting method, the swelling volume principle was applied in a technique particularly designed to evaluate pregelatinized flours and starches. This paper presents data comparing the pasting and swelling-volume methods for evaluation nonprocessed and pregelatinized flours and gives the comparative accuracy of each.

Materials and Methods

In tests with wheat flour only one pregelatinized flour was available. Blends of this flour in varying proportions with an unprocessed wheat flour were evaluated for cold water absorption by the pasting method and by the swelling-volume procedure. An unprocessed white corn flour, an unprocessed yellow corn flour, and three processed corn flours were also tested by both procedures.

The pasting method involved the addition of sufficient water at laboratory temperature to one ounce of flour contained in a 400-ml. beaker. The flour-water paste is stirred by hand with a glass stirring rod $\frac{3}{16}$ inch in diameter to produce a uniform, slowly flowing paste obtained with a time interval of 10 minutes. The absorption number of the flour was calculated on the basis of flour containing 12.5% moisture, and is determined by multiplying the ounces of water used by 100 when the flour weight is one ounce.

In the swelling volume technique 35 ml. of distilled water at 20 C. \pm 2 were added with stirring to 3.5 g. flour contained in a 40-ml. reinforced Pyrex centrifuge tube. The flour-water mixture was stirred

¹ Manuscript received October 3, 1947.

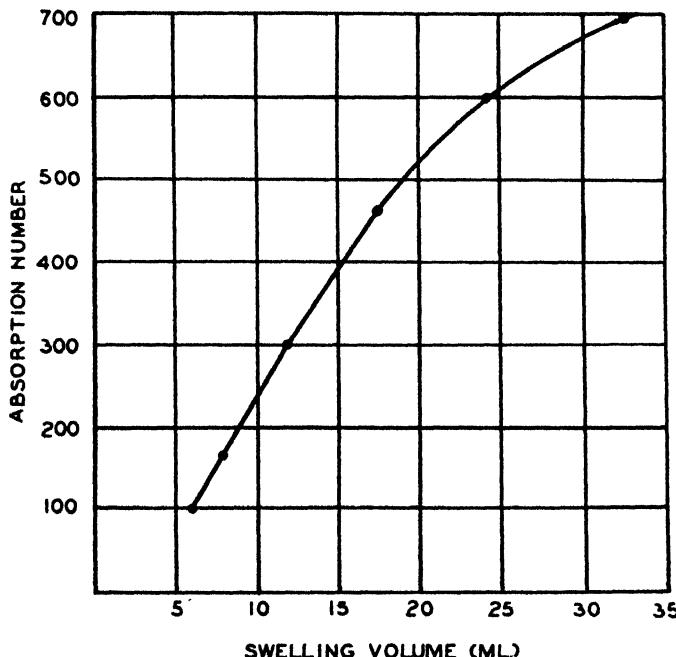
² Research Division, the Griffith Laboratories, Inc., Chicago, Illinois.

for 3 minutes with a $\frac{3}{16}$ -inch glass rod equipped with a rubber tip and then centrifuged for 10 minutes in an International Centrifuge, size 1, Type C, at a speed of 1,300 r.p.m. The resulting volume occupied by the flour is termed "swelling volume."

In preliminary experiments with the swelling volume technique, it was found that stirring the flour-water mixture for 1 and 3 minutes yielded similar results. Allowing the suspensions of unprocessed flours and their blends to stand for periods varying from 4 to 15 minutes before centrifuging had no material effect on swelling volume. In experiments in which the time of centrifuging was varied, the same relative ranking of pregelatinized flour blends was obtained whether 5 or 15 minutes was employed, although the former time yielded higher values.

Results

A comparison of the data for pasting and swelling volume methods when applied to a number of wheat flours is shown graphically in Fig. 1.



The swelling volume method is linearly related to the water absorption number of the pasting method up to a value of about 500, above which the value for swelling increases more rapidly, resulting in a curvilinear relation.

The data in Fig. 1 were obtained by each of three operators. The experimental error of the swelling volume procedure expressed as a percent of the mean did not exceed 8%, whereas the variability in the pasting procedure was as high as 30%.

TABLE I
PASTING METHOD VERSUS SWELLING VOLUME METHOD IN CORN FLOURS

Flour used	Pasting method— water absorption number	Swelling volume method— swelling volume, ml.
Unprocessed white corn flour	130	6
Unprocessed yellow corn flour	145	6
Processed corn flours		
No. 1	280	10.5
No. 2	335	12.5
No. 3	360	13.5

The pasting and swelling volume techniques were applied also to the determination of the cold water absorption capacity of corn flours, the data for which are given in Table I. The swelling volume procedure obviously ranks the flours in the same order as the pasting method.

Literature Cited

1. BRIMHALL, B., and HIXON, R. M. Interpretation of viscosity measurements on starch pastes. *Cereal Chem.* 19: 425-441 (1942).

BOOK REVIEWS

Scientific Principles of Grain Storage. By T. A. Oxley. 103 pp. Northern Publishing Company, Ltd., Liverpool, England. 1948. Price \$3.00.

This little book is the first in the English language which presents in simple terminology a few of the scientific fundamentals underlying good grain storage practice. Within its limited scope, it proves abundantly that a number of problems in grain storage, which have heretofore been dealt with in an empirical manner, are best solved by a rational approach based on recognized principles of physics, chemistry, and biology. It will therefore be welcomed not only by the elevator superintendent as a useful reference manual, but also by the cereal chemist who is interested in basic information.

The English workers have made notable strides in clarifying the physics of grain storage, including such factors as heat conduction and convection and moisture translocation as related to temperature differential. Most American workers in the field have been stressing the biological and biochemical aspects of the problem. This book should be of assistance to these investigators in interpreting their biological data in terms of the physical aspects of the problem.

The subject matter of the 13 brief chapters in the book is as follows: 1, the hazards of grain storage; 2, the physics of a grain bulk; 3, water relations of cereal grains; 4, ventilation of storage places; 5, forced ventilation of bulk grain; 6, drying grain for safe storage; 7, the spontaneous heating of stored grain; 8, methods for measuring temperatures in stored grain; 9, measurement of carbon dioxide and water vapor in the intergranular atmosphere; 10, measurement of insect infestation by carbon dioxide production; 11, insect infestations in bulk grain; 12, the more important grain insects; 13, mites as pests of stored grain. References to the literature are given, but the list is not extensive and is principally in the major field of interest of the author.

The author has made a laudable attempt to demolish several of the many unscientific or experimentally unfounded beliefs which abound in this field. A good ex-

ample of this is his insistence that moisture condensation on grain is unnecessary to promote spoilage, since this may be accomplished simply by an increase in the moisture vapor content in the interseed atmosphere. He also refutes the assumption that bin walls must be permeable to air, and that fresh air is desirable for maximum storage longevity of grain. Where little experimental evidence exists, the author's shrewd observations as well as his scientific objectivity make his conclusions very convincing and his theories excellent hypotheses for the guidance of future research. For example, his theory that the duration of grain viability in storage is governed by its initial content of some unknown factor, which is dissipated at a rate depending upon the intensity of the grain respiration, is indeed attractive. The discussion of the relationship of temperature differential in grain bulks to water vapor movement is a lucid exposition of this very basic phenomenon. The suggestion that measurements of interseed humidity may be useful in predicting incipient deterioration is in line with the conclusions of other workers in the field who believe that the interseed humidity rather than moisture content of the grain initiates and controls deterioration due to fungi.

The discussion of the principles of grain drying focuses attention on the advantages of intermittent drying, which process has recently been developed practically in Europe. The author rightly stresses the scarcity of existing knowledge concerning the influence of various drying conditions and techniques on the qualities of wheat.

Much of the material presented is based on experimentation by the author and his co-workers and it is understandable that his own conclusions and experience are the most thoroughly treated. Frequently only a cursory evaluation of other work is made. For example, the author stresses the importance of a well-developed but unidentified subepidermal mold mycelium in sound wheat which he believes to be responsible for the major portion of grain respiration. Other workers have discounted the importance of this previously developed mycelium both in the respiratory process, and as a factor in storage deterioration and have stressed instead the proliferation under, favorable conditions, of a mycelium from mold spores of other species which exist beneath the seed coat. The data of the author, which show an increase in the respiration of damp wheat with time following harvest, would appear to better support the latter hypothesis.

The first chapter discusses storage hazards in too cursory a manner. A description of deteriorative processes in grain refers to heating as though it preceded molding, sprouting, and rotting. This is incorrect since heating usually follows or accompanies rather than precedes the activity of molds, insects, and biological processes. The listing of means for recognition of deterioration omits the very important factors of odor and discoloration of grain due to heat damage.

In the discussion of hygroscopic moisture equilibria of wheat, it is unfortunate that among the various publications in the field which were cited, no reference was made to the well-known paper of Coleman and Fellows (*Cereal Chem.* 2: 275-287, 1925) which is accepted most widely by American workers as the authority on cereal grain hygroscopicity. The author suggests that hygroscopic equilibrium determinations for wheat might be more valid if measurements were made of intergranular air humidities at equilibrium, after the grain has been brought to various moisture levels. It should be pointed out that such measurements have been made although not widely publicized (*J. E. Anderson, AOM Bull.*, pp. 707-710, 1936, and unpublished data) and they have yielded air humidity data at equilibrium approximately 10% lower at corresponding moisture values than the results obtained by the hygroscopic equilibrium methods. This apparent discrepancy awaits an explanation.

In his discussion of the probable advantages of airtight storage of grain, the author might well have pointed out that no authentic information exists concerning the influence of elevated carbon dioxide concentrations, such as might arise in sealed storage, on the quality of wheat.

The chapter dealing with insects prevalent in grain describes these pests and their habits concisely. The inclusion of illustrations would have increased the practical utility of this chapter. A more notable lack, however, is the complete absence of any discussion concerning fumigants.

This book will prove of value to individuals connected with the buying, storage, shipping, and processing of cereal grains.

MAX MILNER

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Kansas State College
Manhattan, Kansas

Isomerism and Isomerisation. By Ernst David Bergmann. 138 pp. Interscience Publishers, Inc., New York. 1948. Price \$3.50.

This little booklet is an expansion of six lectures given under the general heading of the progress of chemistry. It deals with six subjects of importance to the organic chemist, namely, (I) resonance phenomena in organic molecules, (II) cis-trans isomerism and cis-trans interconversion, (III) isomerisation of olefinic structures, (IV) mechanism of substitution reactions; racemisation and Walden inversion, (V) isomerisation of paraffins and related phenomena, and (VI) mechanism of intramolecular rearrangements.

It is a very interesting and well-written book which brings the reader to the forefront of research in these particular fields of organic chemistry and the theories involved therein. The book contains an excellent literature survey for the specialist and it demonstrates in a striking manner the excellent results which are derivable from the application of physical methods to organic chemistry.

The book was written not with the object of covering the whole field but with the intention of stimulating thought and discussion. This it certainly accomplishes and the reviewer can recommend it both to research students and teachers.

F. SMITH

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Acetylene Chemistry. By Ernst David Bergmann. 108 pp. Interscience Publishers, Inc., New York. 1948. Price \$3.00

This small book like the previous one, *Isomerism and Isomerisation of Organic Compounds*, by the same author is based upon lectures given at seminars dealing with progress in chemistry. This one might well have been called recent advances in the chemistry of acetylene and its compounds.

The book has been written by one well acquainted with organic chemistry and it will prove valuable both to those interested in the general field of organic chemistry and the specialists in acetylene chemistry. For the specialists there is a good literature survey. The progress in acetylene chemistry set forth illustrates in a very striking manner the versatile nature of acetylene and its derivatives which still remains to be exploited.

The first of its three chapters deals with the classification of the reactions of acetylene and the relation between the molecular structure of acetylene compounds and their physical properties; the second discusses the addition reactions of acetylene with ketonic substances; and the third deals with the formation and properties of vinyl- and divinyl-acetylenes and the chemistry of cyclo-octatetraene. The reviewer noted with interest the application and interpretation of the results of physical measurements of acetylene compounds in connection with structural studies.

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Synthetic Methods of Organic Chemistry. A Thesaurus. Volume I. 1942-1944. By W. Theilheimer. 254 pp. Interscience Publishers, Inc., New York and London. 1948. Price \$5.00.

This first volume provides up-to-date methods in organic chemistry which appeared in the literature during the years 1942-1944, inclusive. For each method the number of steps involved and also the yields are quoted. It is pleasing to find that these methods are arranged according to the fundamental reactions of organic chemistry (the way in which organic chemistry is best taught), and not solely according to trivial names or authors' names. The methods are also helpfully subdivided according to the reagents employed and further aid is provided by the alphabetical index which includes names of methods and types of compounds.

The book is intended to serve as a preliminary reference book for laboratory use. Details of the methods can be obtained from the original literature to which reference is made. This book is an extension of Houben's book on methods and Weygand's *Organisch-chemische Experimentierkunst*. New reactions or variations of old ones not to be found in Houben are given either in this volume or will be recorded in the volumes to follow. When the series is brought up-to-date, it will provide an invaluable tool not only to those whose studies were interrupted by the war but to all those engaged in research in organic chemistry.

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EFFECT OF FORTIFICATION OF CANNED BREAD ON STABILITY¹

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ABSTRACT

The stability of thiamine, riboflavin, and niacin was investigated in canned bread during fermentation, baking, and storage. Synthetic and dried yeast sources of enrichment were used and the effect of each on baking quality of the flour and palatability of the finished bread measured. Riboflavin and niacin were retained almost completely during fermentation, baking, and storage. Thiamine, however, regardless of source, was decreased by approximately 15% during baking and 20 and 50% during 6 months' storage at 72° and 100°F. respectively. A 1% level of yeast (flour basis) was preferable to a 3% level insofar as acceptability and baking quality were concerned. The protein quality of the whole wheat and yeast bread was significantly superior to that of the white bread fortified with synthetic vitamins.

During World War II adequate amounts of thiamine, riboflavin, and niacin in rations were provided chiefly through the fortification of the biscuit and cracker components with dried yeast.⁵ The low moisture content of the biscuit component insured against losses of labile thiamine during long periods of storage at elevated temperatures. However, due to the general unpopularity of the biscuits and crackers, endeavors were made to find other stable carriers for these vitamins. Since canned bread was being developed with the intention of partially or completely replacing the biscuit and cracker components, intensive studies were undertaken to investigate the possibilities of this universally well-liked item as a carrier of the B complex vitamins.

Materials and Methods

Fortification of canned bread with synthetic vitamins and natural sources were both investigated. The latter type of enrichment was

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⁵ The dried yeast used in biscuit fortification was specified to contain not less than 600, 600, and 1,000 μ g. of thiamine, riboflavin, and niacin per gram of dried yeast respectively. Such concentrations were obtained by adding the synthetic nutrient to the live yeast cells prior to drying.

preferred as it supplied, in addition to thiamine, riboflavin, and niacin, the lesser known factors of the vitamin B complex, thus producing a more complete and natural food. The natural sources used to provide the B complex vitamins were dried yeast (primary grown or debittered brewers') and whole wheat. Combinations of synthetic and natural sources were also used to determine if the natural enrichment would stabilize the synthetic enrichment.

Study 1. This study was undertaken to survey the potentialities of canned bread as a carrier for the vitamins of the B complex. It was conducted on the first test production run of the three contemplated types to be used in rations, namely, white, white with raisins, and whole wheat bread. Both white types of bread were fortified with synthetic vitamins.⁶ Five cans of each type of bread were assayed initially and at each storage time interval (bimonthly) for thiamine, by the thiocchrome procedure (8), and for riboflavin and niacin by the chemical (3, 6) methods. Stability at 72° and 100°F. was examined over a period of one year.

Study 2. After Study 1 was in progress for several months, it was readily apparent that thiamine stability at 100°F. storage was poor. In order to ascertain the reliability of these data, canned bread was baked in the laboratory⁷ under the most rigidly controlled conditions and again the stability of added thiamine was tested. Dried yeast⁸ (debittered brewers') which had proved so satisfactory in the biscuit and cracker component was also added to canned bread from this batch to study the effect of the yeast on the stability of synthetic thiamine. Preliminary tests to determine the maximum amounts of debittered brewers' yeast which could be employed for enrichment without producing off-flavors showed the 3% level (flour basis) to be the most satisfactory. This study also presented the opportunity of determining baking losses since the doughs were available. Stability tests were made only at 100°F. storage temperature and only two cans of bread were assayed initially and after 3 and 6 months' storage because of the limited number of samples.

Study 3. This investigation was similar to that of Study 2 except that it was carried out on a test production run and the dried brewers'

⁶ Winthrop high potency enrichment tablets were used, one tablet per 50 pounds of flour. The tablets contained 115 mg. of thiamine, 60.5 mg. of riboflavin, 725 mg. of niacin, and 625 mg. of iron per tablet.

⁷ Canned bread was baked by the Cereal and Baked Products Branch. The dough was placed in No. 2 1/2 cans, the cover clinched on, and the bread allowed to proof. Following the proofing stage, the dough was baked in a (450°F.) reel oven containing four trays (24 cans per tray), 96 cans being baked at one time. The cans were removed from the oven after 25-30 minutes, cooled to 190°F. (5 to 10 minutes at room temperature) and then sealed. The basic formula for canned bread in any one study was identical differing only in the type of enrichment made, i.e., substitution of white flour for whole wheat flour or addition of synthetic vitamins instead of dried yeast.

⁸ Dead yeast cells have a deleterious effect on the gluten structure of dough (4). A method of treating the yeast to overcome this gluten-softening effect is essential before the yeast can be used successfully as a fortifying agent. The dry yeast used in Study 2 was not a "treated" yeast. Industry was contacted for the purposes of cooperating in the development of a yeast which would meet the above requirements.

yeast used was "treated" yeast.⁹ Stability of thiamine, riboflavin, and niacin at 72° and 100°F. storage was tested at bimonthly intervals for one year.

Study 4. This extensive study was made on laboratory-baked bread to compare synthetic, whole wheat, and "treated" yeast enrichment on the basis of vitamin B complex stability, contribution of protein¹⁰ and lesser known vitamin factors of the B complex, and palatability. The yeast was used at a 3% level. It was also possible to determine baking losses in each type of bread as well as fermentation losses.

Study 5. In this study other dried yeasts, primary grown and debittered brewers', were used in canned bread at a 1% level and in pan loaf bread at a 3% level to determine whether differences in the baking quality of the resulting bread varied with the different yeasts used. Pan loaves were scored for volume, color, and grain and photographs were taken. Canned breads were stored at 72° and 100°F. and tested for thiamine stability and palatability initially and at 3, 6, 9, and 12 months of storage.

TABLE I
PERCENTAGE LOSS OF B COMPLEX VITAMINS DURING FERMENTATION

Study 4	Thiamine	Riboflavin	Niacin
Synthetic fortification	μg./g.	μg./g.	μg./g.
After mixing	3.62	3.64	38.0
After proofing	3.46	3.59	39.3
Per cent loss	4.00	1.00	+ 2.0
Dried yeast fortification			
After mixing	4.26	3.87	41.8
After proofing	4.07	3.60	39.0
Per cent loss	4.00	4.00	7.0
Whole wheat fortification			
After mixing	3.88	3.14	39.9
After proofing	3.79	3.04	40.5
Per cent loss	2.00	3.00	+ 2.0
Average fermentation loss	3	3	1

Results

Vitamin Stability:

a. Fermentation Losses.

Fermentation losses included those incurred from the time that the ingredients were mixed into a dough until the time the dough was ready

⁹ This yeast was developed by Haffenreffer Yeast Company, Jamaica Plains, Boston, Massachusetts.

¹⁰ The three types of canned bread were evaluated for protein by Dr. P. R. Cannon, University of Chicago, on Committee on Food Research Project NU-3. A detailed report on this study is in press.

to be placed into the oven, a period of approximately three hours. It is seen from Table I that there were no significant losses in thiamine, riboflavin, or niacin in the three types of doughs studied.

TABLE II
PERCENTAGE LOSS OF B COMPLEX VITAMINS DURING BAKING OF CANNED BREAD

Study	Thiamine μg./g.	Riboflavin μg./g.	Niacin μg./g.
<i>Study 2</i>			
Synthetic fortification			
After proofing	9.44	2.62	30.2
After baking	8.12	2.43	31.2
Per cent loss	14.00	8.00	+ 3.0
Yeast fortification			
After proofing	6.65	5.95	55.9
After baking	5.41	5.22	54.7
Per cent loss	19.00	12.00	2.0
<i>Study 4</i>			
Synthetic fortification			
After proofing	3.46	3.59	39.3
After baking	2.72	3.63	41.1
Per cent loss	21.00	+ 1.00	+ 5.0
Dried yeast fortification			
After proofing	4.07	3.52	39.0
After baking	3.26	3.90	39.9
Per cent loss	20.00	+11.00	+ 2.0
Whole wheat fortification ¹			
After proofing	3.79	3.04	40.5
After baking	2.65	3.67	41.1
Per cent loss	30.00	+21.00	+ 1.0
<i>Study 5</i>			
Synthetic fortification			
After proofing	4.69	—	—
After baking	4.36	—	—
Per cent loss	7.00	—	—
Yeast fortification (5 yeasts)			
After proofing	2.16-5.51	—	—
After baking	1.68-4.98	—	—
Per cent loss	10-20	—	—
Average baking loss	16	+ 2	+ 3

¹ Not included in average since baking time was longer.

b. Baking Losses.

Baking losses were determined in three studies. Ten and one-half ounces of dough were placed in a No. 2½ can and white dough baked at 450°F. for 25-30 minutes and whole wheat dough for 40 minutes. Table II shows the percentage loss of thiamine, riboflavin, and niacin which occurred during the baking process.

It is seen that laboratory-baked bread lost approximately 15% of thiamine during the baking process. This figure is in good agreement with those found by others for this baking time (5, 9, 11, 13). The niacin and riboflavin content showed no significant changes on baking. There were no demonstrable differences in the baking losses of synthetically fortified and dried yeast-fortified bread. The thiamine loss in whole wheat bread, however, was somewhat higher due to the longer baking time (30% loss).

c. Storage Losses.

The stability data on canned bread fortified with various sources of the B complex indicated that canned bread was as good a carrier as

TABLE III

STABILITY OF B COMPLEX VITAMINS IN STORED CANNED BREAD, FORTIFIED WITH SYNTHETIC AND NATURAL SOURCES OF ENRICHMENT¹

Source of fortification	Thiamine				Riboflavin				Niacin			
	72°F.		100°F.		72°F.		100°F.		72°F.		100°F.	
	6 mo.	12 mo.	6 mo.	12 mo.	6 mo.	12 mo.	6 mo.	12 mo.	6 mo.	12 mo.	6 mo.	12 mo.
	Per cent retention				Per cent retention				Per cent retention			
Natural												
Dried yeast	84	73	50	31	112	105	105	103	98	94	96	98
Whole wheat	82	75	46	30	88	96	109	100	95	96	93	95
Synthetic												
White	89	75	54	37	114	104	106	109	95	97	96	95
White and bleached raisin	66	60	44	31	100	100	100	100	86	100	90	100
Avg. retention	80	72	49	32	104	101	105	103	93	97	94	97

¹ The retentions in this table represent the average of all storage data taken from Studies 1-5.

ration biscuits for riboflavin and niacin but not for thiamine. Table III summarizes the retention of the thiamine, riboflavin, and niacin in canned bread. Retention of thiamine, riboflavin, and niacin followed the same pattern in the synthetic, yeast, and whole wheat breads. In general, the thiamine content decreased as in other moist carriers (approximately 50 and 20% loss after 6 months' storage at 100° and 72°F. respectively). The riboflavin and niacin content remained at the initial level at both storage temperatures throughout the storage period.

Upon further storage for an additional 6 months, the thiamine content continued to decline but at a slower rate than in the initial 6

months' storage period. The thiamine loss at the end of this time was approximately 70 and 30% at the 100° and 72°F. storage temperatures respectively. The thiamine loss in raisin bread was somewhat greater than in the other types of bread at 72°F. storage, and was in part attributed to the possible action of the bleaching agent used on the raisins.

This pattern of stability characterized by significant decreases in thiamine content with little if any decreases in the riboflavin and niacin content has been reported for other canned foods. The thiamine losses (6 months' storage at 100°F.) for canned meats were found to be higher than for canned bread ranging from 60 to 70% (7). In canned fruits and vegetables the thiamine losses were found to be somewhat lower than in canned bread averaging 40% (1, 10, and 12).

TABLE IV
ANALYTICAL VALUE FOR VARIOUS STORAGE STUDIES, CORRECTED
TO 38% MOISTURE

	Storage time	Thiamine μg./g.		Riboflavin μg./g.		Niacin μg./g.	
		72°F.	100°F.	72°F.	100°F.	72°F.	100°F.
<i>Study 1</i>							
White bread + synthetic vitamins	Initial	1.95	—	2.27	—	29.4	—
	6 mos.	1.97	1.20	2.38	2.51	27.2	28.7
	12 mos.	1.65	0.83	2.23	2.78	27.3	29.3
Raisin bread + synthetic vitamins	Initial	1.08	—	1.69	—	24.7	—
	6 mos.	0.73	0.48	1.57	1.64	21.3	23.6
	12 mos.	0.65	0.33	1.62	1.77	25.0	24.9
Whole wheat bread	Initial	2.09	—	1.56	—	37.9	—
	6 mos.	1.59	0.87	1.42	1.47	37.2	36.7
	12 mos.	1.61	0.64	1.42	1.56	34.8	33.7
<i>Study 2</i>							
White bread + synthetic vitamins	Initial	8.61	—	2.56	—	33.1	—
	6 mos.	—	3.77	—	2.26	—	33.7
	12 mos.	—	—	—	—	—	—
Yeast fortification	Initial	5.75	—	5.54	—	58.1	—
	6 mos.	—	2.60	—	5.22	—	58.8
	12 mos.	—	—	—	—	—	—
<i>Study 3</i>							
White bread + synthetic vitamins	Initial	2.23	—	2.69	—	25.1	—
	6 mos.	1.65	1.20	3.05	3.00	24.8	24.5
	12 mos.	1.42	0.73	3.05	3.21	23.3	21.6
Yeast fortification	Initial	2.82	—	3.11	—	30.9	—
	6 mos.	2.27	1.54	3.40	3.16	34.0	31.9
	12 mos.	1.91	0.82	3.39	3.25	30.3	31.4

TABLE IV—Continued

	Storage time	Thiamine μg./g.		Riboflavin μg./g.		Niacin μg./g.	
		72°F.	100°F.	72°F.	100°F.	72°F.	100°F.
<i>Study 4</i>							
White bread + synthetic vitamins	Initial	2.81	—	3.93	—	41.9	—
	6 mos.	2.64	1.62	4.80	4.64	39.2	38.3
	12 mos.	2.36	1.14	3.92	3.79	41.7	39.8
Yeast fortification	Initial	3.20	—	3.72	—	42.5	—
	6 mos.	2.80	1.73	4.32	4.47	36.2	36.2
	12 mos.	2.50	1.08	4.00	3.82	40.7	39.8
Whole wheat	Initial	2.57	—	3.29	—	40.9	—
	6 mos.	2.27	1.29	3.03	4.08	37.9	37.3
	12 mos.	1.95	0.75	3.25	3.28	41.5	40.0
<i>Study 5</i>							
White bread + synthetic vitamins	Initial	3.89	—				
	6 mos.	3.42	1.77				
	12 mos.	3.07	1.35				
Yeast fortification A.	Initial	4.48	—				
	6 mos.	3.92	2.05				
	12 mos.	3.79	1.40				
B.	Initial	5.22	—				
	6 mos.	4.15	2.49				
	12 mos.	3.86	1.23				
C.	Initial	4.21	—				
	6 mos.	3.39	1.91				
	12 mos.	3.41	1.24				
D.	Initial	3.89	—				
	6 mos.	3.27	2.19				
	12 mos.	3.16	1.55				
E.	Initial	3.06	—				
	6 mos.	—	1.50				
	12 mos.	—	—				

Protein Quality. The protein of the three types of canned bread in study four was evaluated by feeding the dried bread at a 9% protein level to protein depleted adult rats for 14 days and determining the resulting weight recovery at the end of that time (2). The weight gains for rats fed these breads showed that both the whole wheat and the yeast enriched bread was approximately 30% superior in protein quality to the white synthetically enriched bread.

*Evaluation for Lesser Known Vitamin Factors.*¹¹ The presence of the lesser known vitamin factors in the canned breads was determined

¹¹ Evaluation of the canned breads for the lesser known vitamin factors by their growth-promoting value for rats was conducted by Capt. C. French of the Medical Nutrition Laboratory, Surgeon General's Office.

by comparative growth rates of rats fed on equicaloric diets containing 80% bread and 20% basal supplement. The basal supplement contributed adequate amounts of protein, fat, mineral, thiamine, riboflavin, niacin, choline, tocopherol, and vitamins A and D. Growth rate obtained from the three bread diets when compared to a control group receiving Purina Laboratory Chow indicated that no significant rat growth deficiency was involved in any of the three bread diets.

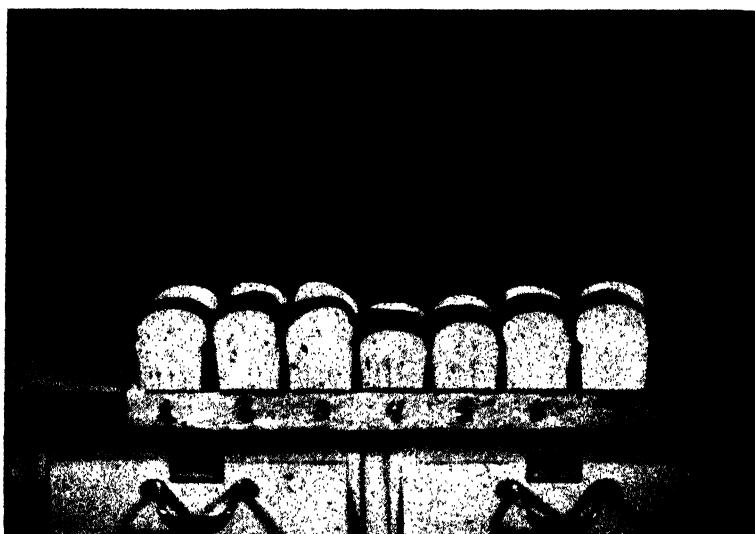


Fig. 1. Effect of 3% yeast addition (flour basis) on bread score.

Bread 1. Primary grown yeast
2. Debittered brewers' yeast (treated to overcome gluten softening effects)
3. Primary grown yeast
4. Debittered brewers' yeast
5. Primary grown yeast
6. Primary grown yeast
7. Control made with enriched flour

*Palatability.*¹² Examinations were made initially and during storage on the bread of Studies 4 and 6. Initially, the 3% yeast bread was judged to be not equal to the synthetically enriched bread in color, grain, and eating quality, and was described as having a bitter, undesirable after taste. The 1% yeast breads were rated as equally as acceptable as the synthetically enriched breads. There were no yeasty or bitter flavors and the color of the grain was lighter than at the 3% level although variations among the yeasts in degree of color produced were evident.

The effects of storage on palatability were more pronounced at the higher storage temperatures. All breads declined in acceptability on

¹² Palatability studies, by taste panels, were conducted by the Food Acceptance Research Branch, QM Food and Container Institute for the Armed Forces.

storage. The 3% yeast bread declined to a greater degree than either the synthetically enriched bread or the 1% yeast bread, the yeasty flavor becoming more apparent and undesirable on prolonged storage. There was no appreciable difference in acceptability of the stored 1% yeast breads and the synthetically enriched breads, thus indicating that the addition of 3% yeast is too high.

Baking Quality. This was determined from the bread score which was made up of individual scores on volume, crumb color, grain, texture, and flavor. The resulting pan loaves made from the six yeasts used at a 3% level (flour basis) are shown in Fig. 1. The amount of gluten softening effect produced by the various yeasts is reflected in the loaf volume. The deleterious effect of the dead yeast cells is especially evident in breads 4 and 5. Bread 2 was made with the treated yeast used in Study 4. The crumb color of all the yeast breads was darker than for synthetically fortified bread (bread 7), breads 4 and 5 having the darkest crumb color. Grain and texture was also scored lowest for breads 4 and 5. Taste tests indicated that the synthetically fortified sample was preferred over the yeast fortified samples with little or no preference for the different yeast breads.

Summary

1. Fortified canned bread was investigated as a possible carrier of the B complex vitamins. Comparisons of synthetic, whole wheat, and yeast enrichment were made for relative vitamin stability and palatability. Fermentation and baking losses were also investigated.
2. There were no significant losses of the B complex vitamins through the fermentation and proofing step. During baking the thiamine content of canned bread was decreased by approximately 15%; the riboflavin and niacin content remained unchanged.
3. During storage at 72° and 100°F. for 6 months the thiamine retention was approximately 80 and 50%, respectively, regardless of the source of enrichment. The use of bleached raisins in white bread increased the losses. Riboflavin and niacin were retained from 90 to 100% for all sources of enrichment at both storage temperatures.
4. There were no deficiencies of the lesser known vitamins in the three types of bread tested (whole wheat, synthetically fortified, and yeast fortified bread) as judged from their growth-promoting values.
5. Yeast and whole wheat bread had better protein value than synthetically fortified bread.
6. A 1% level of yeast fortification was more satisfactory than a 3% level (flour basis), the former producing a bread more nearly equal in color, grain, texture, and taste to bread fortified with synthetic vitamins.

Acknowledgments

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INFLUENCE OF AUTOCLAVING DRY PEAS ON SOME PROPERTIES OF THE PROTEINS¹

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ABSTRACT

The influence of autoclaving dry peas on the peptization and digestibility of the protein has been determined. Autoclaving finely ground dry Alaska peas decreased the protein dispersion by water at pH 1.8 and 10 and by a 1.0 N sodium chloride solution in the pH range 6 to 12. The per cent protein soluble in water and in saline solution was decreased. Mild autoclaving (110°C. for 30 minutes) increased the per cent of alkali-soluble protein, but more drastic autoclaving (130°C. for 60 minutes) again decreased it. The percentage of nitrogenous compounds peptized by different salt solutions was decreased by autoclaving the peas, particularly when drastic autoclaving was employed.

The liberation of amino groups by *in vitro* enzymic digestion of dry peas was decreased by autoclaving as was the liberation of methionine and lysine by *in vitro* trypsin and erepsin digestion. Autoclaving at 130°C. for 60 minutes caused a destruction of 24% of the cystine, 25% of the lysine, and none of the methionine in dry peas as determined by microbiological assay.

The effect of autoclaving soybean oil meal on the proteins has been the subject of considerable study in this laboratory since the observation of Evans and St. John (7) that the poor nutritive values of some commercial soybean oil meals was caused by overcooking. It had previously been shown by other investigators that cooked soybean oil meals were better nutritionally than raw meals (8, 9). Subsequent work has been conducted to cast more light on the nature of the effect of heat-treatment on soybean oil meal proteins.

Soybean oil meal proteins have been known for some years to behave differently from most other proteins with regard to the effect of heat on nutritive value (12). It was believed desirable to study the influence of autoclaving on other proteins than those of soybean oil meal. Eggs and dry peas were chosen. The data on eggs are presented elsewhere (14). Although peas are a legume, as are soybeans, the nutritive value of dry peas is not improved by cooking (17). Because of this difference between dry peas and soybeans and because

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of the economic importance of dry peas in eastern Washington, they were chosen for the present investigation.

Materials and Methods

A sample of finely ground dry Alaska peas⁴ was divided into three portions. One portion was not autoclaved, one was autoclaved for 30 minutes at 110°C., and the other was autoclaved for 60 minutes at 130°C.

Peptization studies on the three samples were carried out at different pH levels using hydrochloric acid and sodium hydroxide to adjust the pH as described by Evans, Henry, and St. John (4). A similar investigation was made by peptizing with 1.0 N sodium chloride solution at different pH levels (4) and six different salts at the normal pH of the salt solutions.

To determine the influence of autoclaving on protein solubility, water-soluble nitrogen, nitrogen soluble in saline solution, alcohol-soluble nitrogen, alkali-soluble nitrogen, and residual nitrogen, determinations were made on each of the three samples by the method of Lund and Sandstrom (11).

In vitro enzyme digestions of the three pea samples were carried out as described by Evans (3). Pepsin, trypsin, trypsin followed by erepsin, and pepsin followed by trypsin followed by erepsin were the enzymes used in this study.

To determine methionine, cystine, and lysine in the dry peas two methods of hydrolysis were used to liberate the amino acids. In the first, 2.0-g. samples of dry peas were hydrolyzed by autoclaving at 15 pounds pressure for 8 hours with 10 ml. of 10% hydrochloric acid in a sealed tube. For the second method 2.0-g. samples of the dry peas were digested with the enzymes trypsin and erepsin as described by Evans (3). Amino nitrogen was determined by the Van Slyke method (16), methionine with *Lactobacillus arabinosus* (15), lysine with *Streptococcus faecalis* (1), and cystine in the acid digest with *L. arabinosus* (15), and in the enzyme digest with *Leuconostoc mesenteroides* (15) using entirely synthetic media.

Results and Discussion

Protein Solubility in Various Reagents. Evans and St. John (7) observed that a very pronounced change in the solubility of the proteins in raw soybean oil meal resulted from autoclaving. There was a decrease in the proteins soluble in saline solution, an increase followed by a decrease in the alkali-soluble proteins, and an increase in

⁴ The dry Alaska peas were kindly furnished by E. A. Dumas of the Klemgard Pea Processing Company of Pullman, Washington.

TABLE I
INFLUENCE OF AUTOCLAVING DRY PEAS ON PROTEIN SOLUBILITY
IN VARIOUS REAGENTS

Protein fraction	Not autoclaved	Autoclaved at 110°C. for 30 min.	Autoclaved at 130°C. for 60 min.
Water soluble (%)	59.1	25.3	21.8
Soluble in saline solution (%)	20.6	16.3	3.2
Alcohol soluble (%)	1.6	2.9	2.8
Alkali soluble (%)	5.2	29.4	6.7
Residual (%)	14.3	27.3	66.3

the residual protein. Similar data for dry peas are presented in Table I. As with soybean oil meal, autoclaving caused a decrease in the protein fractions soluble in water and in saline solution and an increase in the residual protein fraction. Autoclaving at 110°C. for 30 minutes increased the amount of protein in the alkali-soluble fraction. Autoclaving at 130°C. for 60 minutes resulted in a low alkali-soluble protein fraction again. These results are all in agreement with those previously reported for soybean oil meal.

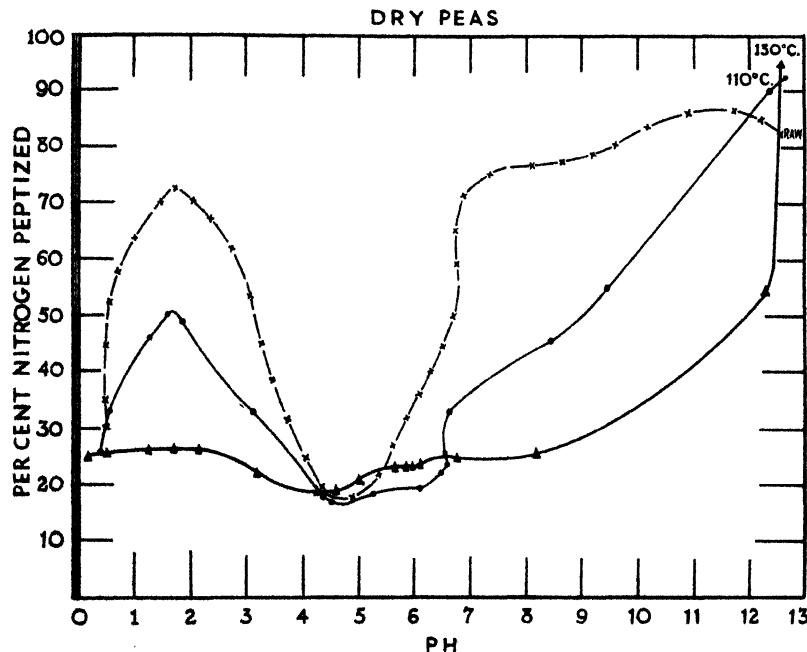


Fig. 1. The influence of autoclaving dry peas on the peptization of the nitrogenous constituents by solutions of different pH values.

Raw peas

Peas autoclaved for 30 min. at 110°C.

Peas autoclaved for 60 min. at 130°C.

X—X

0—0

Δ—Δ

Although no feeding studies were conducted, the data presented here for the alkali-soluble proteins taken with the work of Woods, Beeson, and Bolin (17) indicate that there would be no relationship between the per cent of protein in the alkali-soluble fraction and the protein nutritive value of dry peas such as was observed with soybean oil meal by Evans, McGinnis, and St. John (6). Woods, Beeson, and Bolin (17) observed that an increasingly harmful effect on the nutritive value of dry peas for rats resulted from increased heating as contrasted to the improvement which first occurs in soybean oil meal.

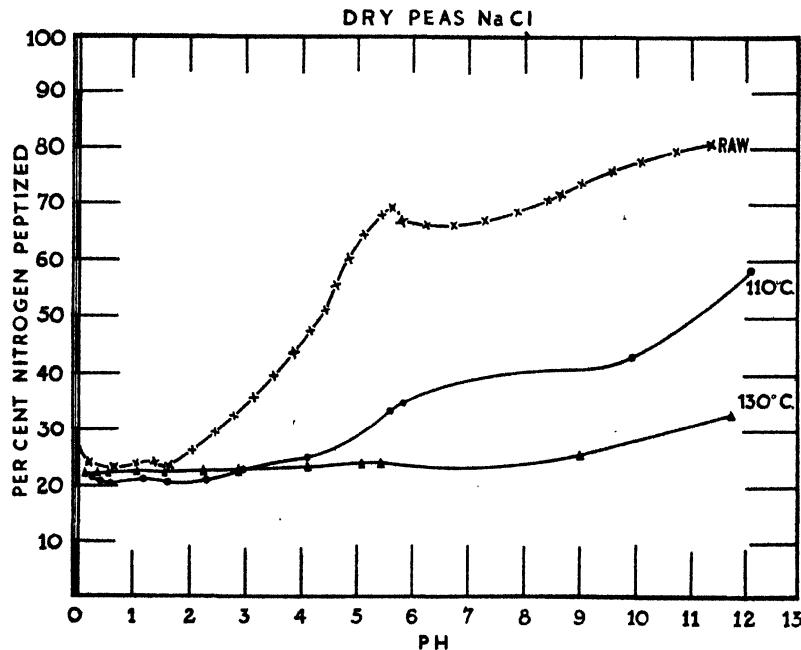


Fig. 2. The influence of autoclaving dry peas on the peptization of the nitrogenous constituents by 1.0 N sodium chloride solution of different pH values.

Raw peas
Peas autoclaved for 30 min. at 110°C.
Peas autoclaved for 60 min. at 130°C.

X—X
○—○
△—△

Peptization of Protein at Different pH Levels. To investigate further the influence of autoclaving on protein dispersion, peptization curves were obtained using as peptizing agents water or 1.0 N sodium chloride solution, the pH of which was adjusted to different values with sodium hydroxide or hydrochloric acid. The results are presented in Figs. 1 and 2. Evans, Henry, and St. John (4) earlier obtained a clear-cut peptization curve with dry peas using hydrochloric acid and sodium hydroxide to adjust the pH of the suspensions. They also

obtained an interesting curve using a 1.0 *N* sodium chloride solution. It was believed desirable to determine the influence of autoclaving dry peas on the shape of these curves. Points of maximum dispersion on the hydrochloric acid-sodium hydroxide curve were at pH 1.8 and 12.3. Minimum dispersion occurred at pH 4.6. Peptization at pH 1.8 was decreased by autoclaving the peas at 110°C. for 30 minutes. It was further decreased by autoclaving at 130°C. for 60 minutes until it was little greater than that occurring at the point of minimum peptization.

At the point of minimum peptization there was little difference in the per cent of protein peptized from the raw and the different heat-treated peas. Since the 18% of nitrogenous compounds soluble at pH 4.6 was not decreased by autoclaving, this fraction is either non-protein in nature, or belongs to some protein group that is not denatured by the heat treatment here used.

TABLE II
INFLUENCE OF AUTOCLAVING DRY PEAS ON PROTEIN SOLUBILITY
OF THE PROTEINS IN 1.0 N SALT SOLUTIONS

Salt	Not autoclaved		Autoclaved at 110°C. for 30 min.		Autoclaved at 130°C. for 60 min.	
	% N		% N		% N	
	pH	Peptized	pH	Peptized	pH	Peptized
NaCl	5.7	66.7	5.8	31.8	5.3	24.0
Na ₂ SO ₄	5.8	69.4	5.9	32.2	5.4	23.6
CaCl ₂	5.0	61.1	5.1	34.1	4.8	23.8
AlCl ₃	3.3	35.3	3.6	22.0	3.6	20.4
Na ₃ PO ₄	11.1	69.0	11.4	51.6	11.4	30.0
Li ₂ SO ₄	5.5	69.0	5.6	35.1	5.2	25.4

Autoclaving at 110°C. decreased the amount of protein peptized at pH 7.0, which would be considered a relatively mild treatment, to almost as low a value as for the peas autoclaved at 130°C., which was little greater than at the point of minimum dispersion.

At pH 12.5 the nitrogenous substances from all of the pea samples were over 80% peptized. This might have resulted from a breakdown of the protein by the strong alkali.

Nelson (14) conducted a similar study with eggs. Results with egg yolk proteins were in most respects similar to those with dry peas; boiling for 10 minutes reduced the solubility of the nitrogen compounds to about 4% at pH values below 9. Boiling decreased the amount of nitrogenous compounds in egg yolk, which were soluble at the point of minimum peptization, in contrast to the data with peas. However,

the dispersion characteristics of egg yolk proteins and peas are influenced similarly by heat. The solubility of egg albumen was decreased from above 90% in the range pH 1-12 for the raw albumen to 10% of the albumen of the eggs boiled for 30 minutes.

Peptization of Proteins by Different Salt Solutions. Autoclaving dry peas decreased the dispersion of the proteins in 1.0 N solutions of six different salts. Autoclaving at 130°C. gave a greater decrease than at 110°C. (Table II). The pH values of the suspensions were generally slightly lower for peas autoclaved at 130°C. than for raw peas.

Liberation of Amino Nitrogen by Proteolytic Enzymes. The results of enzyme digestion studies are presented in Table III. In all cases, autoclaving decreased the extent of digestion, the decrease being more pronounced the higher the temperature and the longer the time of autoclaving. Evans (3) observed an increased liberation of amino groups by trypsin or trypsin and erepsin after autoclaving raw soybean oil meal for 30 minutes at 110°C., while a decreased liberation followed

TABLE III
INFLUENCE OF AUTOCLAVING DRY PEAS ON LIBERATION OF AMINO
NITROGEN BY PROTEOLYTIC ENZYMES
(PER CENT OF TOTAL NITROGEN LIBERATED)

Enzymes used	Not autoclaved	Autoclaved at 110°C. for 30 min.	Autoclaved at 130°C. for 60 min.
Pepsin	17	16	11
Trypsin	53	45	31
Trypsin-erepsin	46	40	34
Pepsin-trypsin-erepsin	38	39	35

the 130°C. for 60 minutes autoclaving treatment. The peptide bonds of raw peas were hydrolyzed by trypsin or trypsin and erepsin to a greater extent than those of peas autoclaved for 30 minutes at 130°C. Thus, the proteins of soybean oil meal are rendered more digestible and those of peas less digestible by the mild autoclaving treatment. Since the beneficial results with soybeans are attributed to a destruction of the trypsin inhibitor present, it may be inferred that a trypsin inhibitor is not present in dry peas; actually none was found in peas by Borchers *et al.* (2). The differences in digestibility might well account for the differences in nutritive value between soybeans and peas which result from autoclaving. Evans, McGinnis, and St. John (6) observed a close relationship between *in vitro* trypsin and erepsin digestion and chick digestion of soybean oil meal proteins.

Nelson (14) studied the influence of boiling on the *in vitro* enzymic digestion of the proteins of egg yolk and egg albumen. Egg albumen behaved in much the same manner as soybean oil meal, since boiling

for 30 minutes increased the extent of hydrolysis by trypsin or trypsin and erepsin. Egg whites contain a trypsin inhibitor, which has been identified with ovomucoid (10) which accounts for the similarity of behavior between soybeans and egg whites. On the other hand, egg yolk proteins behaved more like those of dry peas because the 30-minute boiling decreased trypsin and erepsin digestion.

Methionine, Cystine, and Lysine Liberation by Acid and by Enzyme Digestion. McGinnis and Evans (13) observed that soybean oil meal which had been autoclaved at 130°C. for 60 minutes gave normal growth with chicks when the chick diet was supplemented with 0.5% each of methionine, cystine, and lysine, but not otherwise. They,

TABLE IV

INFLUENCE OF AUTOCLAVING DRY PEAS ON LIBERATION OF METHIONINE, CYSTINE, AND LYSINE BY ACID AND BY *in vitro* TRYPSIN AND EREPSIN DIGESTION

Determination	Method of hydrolysis	Not autoclaved		Autoclaved at 110°C. for 30 min.		Autoclaved at 130°C. for 60 min.	
		In peas	Enzyme acid	In peas	Enzyme acid	In peas	Enzyme acid
Amino nitrogen	Acid	% ¹	%	% ²	%	% ²	%
	Enzyme	2.65		2.65		2.56	
Methionine	Acid	0.31		0.29		0.36	
	Enzyme	0.12		0.11		0.08	
Cystine	Acid	0.16		0.17		0.13	
	Enzyme ¹	(0.53)	(331)	(0.54)	(318)	(0.43)	(331)
Lysine	Acid	1.56		1.60		1.20	
	Enzyme	0.99		0.75		0.53	

¹ Partial digests of soybean oil meal give a stimulating cystine action for the organisms *Lactobacillus arabinosus* and *Leuconostoc mesenteroides* on the synthetic type of media used which was greater than could be accounted for if all the sulfur in the soybean oil meal were cystine. The value for cystine is considerably higher for the enzyme digest than for acid ones, and the enzyme values are given here for comparative purposes only.

² Per cent on a basis of total weight of the dry peas.

therefore, concluded that the deficiencies of overcooked soybean oil meal could be accounted for by inactivation or methionine, cystine, and lysine. For that reason the influence of autoclaving on the amino nitrogen, methionine, cystine, and lysine content of dry peas, both after acid and enzyme hydrolysis, was determined with the results shown in Table IV. The value after acid hydrolysis supposedly represents total, and that after *in vitro* trypsin and erepsin digestion, available amino acids.

Although no loss of total methionine occurred, losses amounting to 24% of the cystine and 25% of the lysine were caused by the autoclaving the peas for 60 minutes at 130°C. Evans and McGinnis (5)

observed similar results from the more drastic autoclaving of soybean oil meal. The slight decrease in amino nitrogen resulting from the more drastic autoclaving was in all probability caused by the destruction of cystine and lysine.

There was a progressive decrease in the per cent of amino nitrogen, methionine, and lysine liberated from dry peas by *in vitro* trypsin and erepsin digestion after increasingly drastic autoclaving treatment. The results from more drastic autoclaving are similar to observations of Evans and McGinnis (5) with soybean oil meal. Although a similar decrease did not occur in cystine availability, the data are only suggestive, since the values obtained after enzyme digestion were over three times as high as the total cystine determined on the acid digest. Both *Leuconostoc mesenteroides* and *Lactobacillus arabinosus* give high results for cystine in partially hydrolyzed soybean oil meal or dry peas on the type of media used. Apparently the partially hydrolyzed meals stimulate these organisms on such synthetic media. Prolonged acid hydrolysis often results in considerable losses of cystine, so that the total cystine values may be low. At least the results are suggestive, even though not conclusive, that autoclaving at 130°C. did not reduce the percentage of cystine present that was liberated by *in vitro* trypsin and erepsin digestion. Similar results have been obtained with *in vitro* pepsin, trypsin, and erepsin digestion of soybean oil meal. No increased enzymic liberation of methionine, cystine, and lysine resulting from the mild autoclaving of dry peas was observed such as occurred with raw soybean oil meal (5).

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EVALUATION OF THE BAKING PROPERTIES OF . ROLLER PROCESS NONFAT DRY MILK SOLIDS BY A FARINOGRAPH PROCEDURE¹

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and GASTON DALBY²

ABSTRACT

A farinograph procedure for evaluating the quality of nonfat dry milk solids for breadmaking is described. The procedure involves a determination of the absorption of a mixture of equal parts of spring wheat flour and nonfat milk solids after 10 to 14 minutes of mixing to allow time for any softening action of the milk solids on the gluten to take place. From a knowledge of the absorption of the flour itself, the absorption due to the milk solids can be computed. The absorption as measured by this procedure is a summation of two factors: the actual physical absorption of water by the dry milk plus a measure of its softening action. Nonfat milk solids of satisfactory baking quality give absorption values of 70% or higher, while solids of low quality give values of 65% or lower.

Ever since the introduction of nonfat dry milk solids into the baking industry the evaluation of their baking properties has presented difficulties. A sample with poor baking properties in commercial production may, and in most cases does, give reasonably good laboratory baking results. To overcome this anomaly, it is customary in experimental baking practice to increase the percentage of milk based on the flour to as high as 12%. Extra punishment of the dough such as repeated molding has also been tried. No definitive simulation of shop conditions which will show up a milk of intermediate value has yet come into general use.

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² Ward Baking Company, New York, N. Y. With the technical assistance of Robert G. Scherf.

Many laboratories have measured the viscosities of mixtures of flour, milk, water, and some correlation with baking properties of the milk has been obtained. Since the introduction of dough testing apparatus many efforts have been made to measure a softening effect of milk on flour. Usually 6% to 10% dry milk based on the flour is used. Skovholt and Bailey (6) obtained a positive correlation between dough plasticity and baking properties of dry milks. The practical difficulty in these earlier studies has been the lack of a clear-cut evaluation of nonfat milk solids of intermediate baking properties.

Under shop conditions nonfat milk solids of poor baking quality result in soft, slack doughs, prolonged proofing time, and poor oven spring. The bread has small loaf volume and poor crumb and texture. Hence all known facts suggest a definite correlation between "baking quality" of milk solids and dough consistency.

The presence of increasing quantities of milk should magnify this effect. To test this theory, preliminary Brabender Farinograph studies were made with two milks, one of which gave good commercial results while the other gave very poor commercial results. The proportions of flour and nonfat milk solids in the mix were varied from one extreme to the other in 10% increments and the most pronounced result was obtained with a 50-50 mixture of flour and milk solids.

Method

The absorption of a spring wheat flour is determined by titrating a 300-g. sample ("as is" basis) with water until a maximum dough development of 500 Brabender Units is obtained. Place 150 g. of the flour, 150 g. of the nonfat milk solids in the farinograph mixer, and add the amount of water computed as follows: Let F be the absorption of the flour in percentage based on the original titration of the flour.

Then the percentage water to be added to the mixture will be: $\frac{F + 65}{2}$.

(Thus an absorption of 65% is arbitrarily assigned to the milk.) An average sample of nonfat milk solids when mixed with flour will reach the 500 line immediately, but within 30 seconds or so the mixture will soften. After 10 to 14 minutes mixing the pointer will again reach the 500 line. Then add more water in 1.5 ml. portions, to hold the pointer on the 500 line during further mixing until maximum absorption is measured. Let X be the final absorption of the mixture; the absorption of the nonfat milk solids is $2\left(X - \frac{F}{2}\right)$. Record two factors: 1.

Development period (the time required in minutes for the mixture to reach a consistency of 500 with the original addition of water). 2. Final absorption of milk.

Results and Discussion

Using one sample of nonfat milk solids, tests with strong spring patent and weak Southwestern patent flours have given essentially the same results. This test has been repeated with milks of various degrees of quality. The type of flour used in the test is not, therefore, a critical factor. A sample of commercially ground roller process nonfat milk solids was screened into three particle size fractions. Farinograph tests on each fraction were similar. This procedure was also repeated with milks of various degrees of quality. Particle size had no effect.

Using the above method with nonfat milk solids of poor, intermediate, and good baking quality and a flour of 63.0% absorption, the curves shown in Fig. 1 were obtained. The absorption of the poor milk was less than 65%, since the consistency of the mixture never reached 500 Brabender Units. The development period is recorded

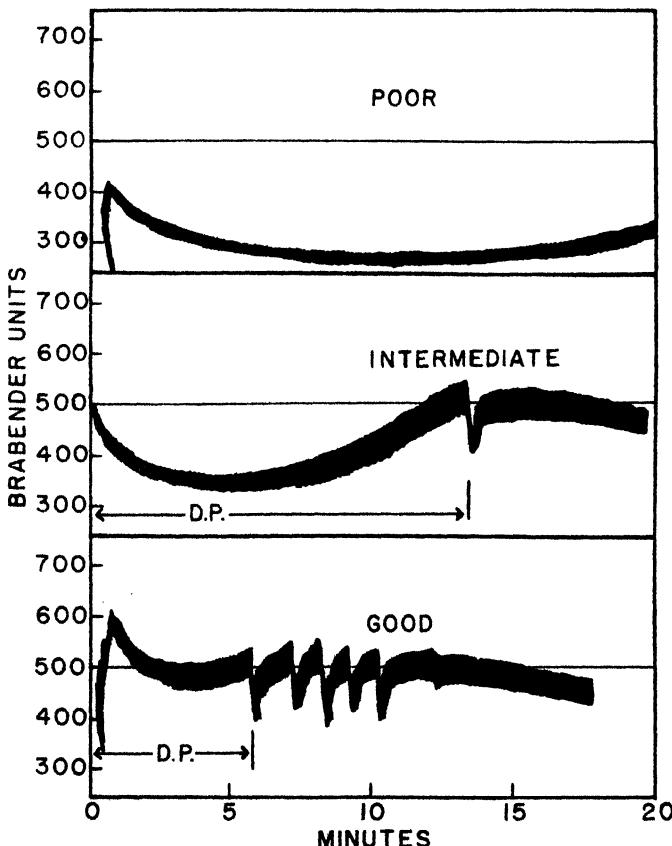


Fig. 1. Farinograph charts for equal mixtures of flour and poor, intermediate, and good quality nonfat milk solids, respectively.

as "never reached." The sample of intermediate quality gave a development period of 12.5 minutes and a calculated final absorption of 67%. The nonfat milk solids of good baking properties had a final absorption of 76.5%. The development period was 6 minutes.

The absorption figure obtained by this determination is not entirely a measure of the actual absorption of the milk itself, but a summation of the softening action of the milk on the flour and the actual physical absorption of the milk. This figure is of paramount interest to the baker.

The development time, in theory, depends upon an equilibrium. On mixing the flour, the gluten undergoes development, the rate of which is influenced by the properties of the nonfat milk solids and is, therefore, a useful index of the quality of the milk.

A sample of nonfat milk solids of poor baking quality was tested in the usual manner and the chart made. Then 150 g. of flour were

TABLE I
FARINOGRAPH ABSORPTION VALUES OF EQUAL MIXTURES OF FLOUR AND
NONFAT MILK SOLIDS OF VARYING QUALITY ADDED BEFORE
AND AFTER GLUTEN DEVELOPMENT

Quality	Absorption ¹ Milk solids added	
	Before gluten development %	After gluten development %
Poor ²	Less than 65	67
Intermediate ²	67	72
Good ²	76.5	80

¹ Absorption figures are those calculated for the milk solids, and are not the absorption values of the mixture of flour and milk solids.

² Same milk samples from which the curves in Fig. 1 were obtained.

placed in the farinograph, the amount of water needed for the 150 g. of flour added, and the dough mixed for the normal time required for the particular flour to reach its mixing peak. (The chart did not read a consistency of 500 since insufficient total dough was in the mixer to read normally.) The machine was stopped and 150 g. of the milk solids and 97.5 ml. of water added and the mixing was resumed (97.5 ml. of water is equal to 65% absorption on 150 g. of milk solids). Since the gluten had an opportunity to develop by itself before the milk solids were added, the effect of the milk on gluten development was not nearly so marked.

As shown in Table I there is considerable improvement in the absorption value of a poor quality milk when it is added to the flour after the gluten has developed. The increase in absorption of an intermediate quality milk is not so great, and in the case of the good quality

milk is still proportionately less. These data lend some support to the theory that a milk poor in baking quality affects the dough by interference with gluten development.

It has been known for many years that heat treatment of milk was necessary for good baking properties. Geddes and his associates (1), Harland and Ashworth (2), and, earlier, Skovholt and Bailey (5) have shown that the adverse effect of a poor milk is due to its content of undenatured serum protein which depends upon the time and temperature of preheat treatment. Measurement of the percentage of undenatured serum protein present in a sample of nonfat milk solids should, therefore, serve as a useful index of its quality for baking. In fact, Ashworth (2) has published a technique for this purpose. Geddes and associates (1) have suggested that the Rowland procedure (3, 4) for determining the nitrogen distribution in milk might prove valuable for the evaluation of milk for bakery purposes.

Six milks of various degrees of baking quality have been tested by the farinograph procedure and by the methods proposed by Geddes (1)

TABLE II
COMPARISON OF GEDDES,¹ ASHWORTH, AND FARINOGRAPH
PROCEDURES WITH QUALITY

GEDDES PROCEDURE ¹		
Sample number	Undenatured serum protein N Per cent of total N	Rating
1	5.37	Good
2	5.50	Good
3	6.63	Good
4	10.63	Poor
5	8.14	Good
6	9.57	Poor
ASHWORTH PROCEDURE		
	Whey protein N (mg. per g.)	
1	0.5	Good
2	0.0	Good
3	0.8	Good
4	1.1	Good
5	1.7	Poor
6	1.4	Good
FARINOGRAPH PROCEDURE		
	Development period	Absorption %
1	8½ minutes	71½
2	7½ minutes	76
3	Never reached	Less than 65
4	Never reached	Less than 65
5	0 minutes	82
6	Never reached	Less than 65

¹ The Geddes application of the Rowland procedure for the evaluation of milk solids for bakery purposes.

and by Harland and Ashworth (2). The results are shown in Table II. Commercial results check best with the farinograph procedure ratings.

The Geddes adaptation of the Rowland procedure and the Ashworth technique may be of great value in the study of milk processing as well as to the baker. The farinograph procedure, however, enables the laboratory technician to determine the effect of nonfat milk solids on gluten development and on absorption. A comparison of commercial results (based on hundreds of tests) with the Brabender Farinograph procedure permits the following tentative ratings as shown in Table III.

TABLE III
TENTATIVE QUALITY RATINGS OF MILKS FROM
RESULTS OF FARINOGRAPH TESTS

Development period	Absorption %	Rating
Under 10 minutes	Over 70	Excellent
10 to 12 minutes	Over 70	Good
12 to 16 minutes	Over 70	Fairly good
12 to 16 minutes	67-70	Fair
Over 16 minutes	Under 67	Poor

Although comparatively few tests with the farinograph procedure have been carried out on nonfat milk solids made by the spray process, the results indicate that the test holds equally as well as for roller process nonfat milk solids. The only modification necessary in the procedure is the use of an original 40% absorption on the milk rather than the 65% absorption figure taken for roller process milks. Since no commercial testing has been done with spray milks to correlate results with the farinograph procedure, the ratings given in Table III do not apply to spray process nonfat milk solids.

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THE DISPOSITION OF LIPASE AND LIPOXIDASE IN BAKING AND THE EFFECT OF THEIR REACTION PRODUCTS ON CONSUMER ACCEPTABILITY¹

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ABSTRACT

Data are presented on the lipase activity of certain wheat fractions and bread constituents. Lipoxidase assay values for several wheat products, enzyme preparations, and baking ingredients also are listed.

The lipase and lipoxidase activity of normal baking ingredients, as well as that present in such enzymatically active adjuncts as soybean meal and wheat germ, is completely inactivated during the baking process. These enzymes are thus nonfunctional during the storage of baked goods, and any hydrolytic or oxidative rancidity occurring in such products must be due to chemical hydrolysis and autoxidation, respectively.

The products of lipolytic action have a detrimental effect on loaf characteristics. Unsaturated fatty acids are more detrimental to loaf characteristics than are saturated fatty acids; however, both types exert a marked detrimental effect on consumer acceptability in concentrations as low as 0.5 of 1%. Oxidized shortenings do not affect baked loaf characteristics appreciably but they do affect consumer taste reactions adversely. The release of fatty acids and the oxidation of lipid material by enzyme action during storage of flour, however, constitute an important economic problem.

Available data indicate that both hydrolytic and oxidative rancidity occur in flour and other wheat products as the result of lipase and lipoxidase action. In baked products rancidity is believed to be due mainly to autoxidation, although no experimental proof of this is available in the literature.

The distribution of lipase in wheat has been investigated by Sullivan and Howe (15), Pett (10), and Engel (5). In addition to the known lipase activity of flour and other wheat fractions, various baking adjuncts are also known to possess lipolytic activity. Van Laer (18) reported the presence of lipase in malt extract, and Gorback and Günther (6) observed the same enzyme in yeast. Certain possible baking adjuncts including wheat germ and soybean flour also contain large amounts of these enzymes.

The literature concerning the relation of esterases in milling and baking has been reviewed recently by Sullivan (14). If present in sufficient quantities the lipolytic enzymes may have detrimental effects

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on the bread product due to the free fatty acids released from either the added shortening or the natural lipid material of flour prior to baking. Barton-Wright (2) found that doughs made from flour stored at high moisture levels and relatively high temperatures lacked extensibility. The volume, flavor, and taste of the bread also were inferior. Sullivan, Near, and Foley (16) and Barton-Wright (2) reported that small amounts of unsaturated fatty acids such as oleic, linoleic, and linolenic acid did not injure the breadmaking characteristics to the extent that might be expected from the deleterious effect on gluten. Sullivan *et al.* (16) showed that the damaging action on breadmaking occurred only when the unsaturated acids became rancid or were oxidized either by air or by the lipoxidase enzymes in flour. The oxidized unsaturated fatty acids produced a dough which was "dead" and a bread with poor volume, flavor, and taste.

The presence of the enzyme lipoxidase has been demonstrated in wheat germ although the quantity is only 2.5% of that found in soybean meal (Sumner, 17). No data are available concerning the lipoxidase content of patent flour. This enzyme is important in dealing with foodstuffs because of its accelerating influence on the development of oxidative rancidity in fat and its destruction of certain vitamins. Its carotene destroying power has been the subject of a process patented by Haas and Bohn (7) to bleach bread dough.

Practically all the work on lipoxidase has been done with soybean meal. It has been found that this enzyme oxidizes higher unsaturated fatty acids such as linoleic and linolenic acid. These acids combine with at most one or two moles of oxygen, respectively, in the enzymatic oxidation. Bergström (3) found that the oxidation of linoleic acid with soybean lipoxidase followed the same general course as a metal catalyzed autoxidation of this acid. Both the metal and enzyme catalyzed reaction products exhibited maximum ultraviolet light absorption at $232\text{ m}\mu$ and the same two monohydroxy acids, 9- and 13-hydroxystearic acid, were isolated in comparable yields from the hydrogenation products. Higher oxygenated acids were also formed in comparable amounts by enzymatic and autoxidation reactions.

The present study was undertaken to establish the disposition of lipase and lipoxidase enzymes in baked products and to determine the threshold value for the enzyme reaction products in baked bread by means of consumer acceptability studies.

Materials and Methods

The flour used in this study was a blend of experimentally milled flours having a protein content of 13.0% and an ash content of 0.44%.

The special shortenings used were COVO shortening and a hydrogenated cottonseed oil.

The straight dough procedure employed by Johnson and Miller (8) was followed. All loaves were baked in duplicate. Bread to be used for subsequent organoleptic testing was wrapped in wax paper, sealed with melted paraffin, and stored at $77^\circ \pm 1^\circ\text{F}$. until needed.

The method for determining lipase activity adopted for this study was based on the manometric determination of the carbon dioxide evolved from a bicarbonate buffer (pH 7.4) as a result of the liberation of free fatty acid from mono-n-butyryl substrate (Singer and Hofstee, 13). This procedure adopts several compromise conditions. Although the higher triglycerides of fatty acids would provide a natural substrate system, these agents are only slightly soluble in water and therefore difficult to manipulate. Mono-n-butyryl substrate is soluble in water in all proportions. The pH employed (13) is somewhat higher than the optimum of 5.2 for wheat lipase acting on higher triglycerides (Sullivan and Howe, 15) and is also well above the normal pH of 4.6-5.3 found in flour doughs. For comparative purposes, however, the results obtained under the conditions of the experiment were entirely satisfactory. The lipase enzyme preparation was prepared routinely immediately prior to analysis by extracting the source for 15 minutes at 3°C . with 10 volumes of water, followed by centrifugation at 1800 r.p.m. for 5 minutes and adjustment of the pH within the range 6.6-6.8. Ground defatted wheat germ was used as the source of active lipolytic enzymes.

Lipoxidase activity was determined by the method of Reiser and Fraps (11) as modified by Mitchell and King (9). A stock carotene solution was prepared by dissolving 50 mg. of purified carotene (11) in 250 ml. of doubly distilled acetone. The carotene substrate solution consisted of a 50 ml. portion of this stock solution plus 60 mg. of fresh Wesson oil brought to 100 ml. volume with purified acetone. These solutions were stored at -23°C . Before withdrawing aliquots, the solutions were heated on a steam cone to dissolve precipitated carotene and subsequently cooled to room temperature. The buffer solution consisted of 59.4 g. disodium phosphate dodecahydrate and 22.7 g. monopotassium phosphate (anhydrous) per liter of solution (pH 6.5). A reaction temperature of $30^\circ \pm 0.1^\circ\text{C}$. was maintained. Enzyme suspensions were made by grinding the source material in a glass mortar with a small quantity of water. After adjustment to volume the suspension was centrifuged, filtered, and analyzed for lipoxidase activity. Ground defatted soybean meal was used as a source of potent lipoxidase activity.

The saturated and unsaturated fatty acids of cottonseed oil were separated from each other by fractional crystallization. The cottonseed oil was saponified with alcoholic potassium hydroxide, acidified with hydrochloric acid, and the fatty acids extracted with Skellysolve F. Separation of the saturated fatty acids was effected at -23°C . The unsaturated fatty acids which remained in solution were freed from solvent under vacuum.

Selected shortenings were oxidized artificially by passing a steady stream of undried, filtered air through the fat which was heated to 90°C . with infra-red lamps. Samples were taken at arbitrary intervals for the determination of peroxide numbers (Wheeler, 19).

Organoleptic tests were made using six experienced judges. Tasting sessions were held at the same time and place each day and each judge was given a freshly cut slice of each bread type to which a coded number was assigned. Samples were rated independently for aroma and flavor acceptability and assigned scores ranging from 1 to 10.

Results and Discussion

The Lipase Activity of Baking Ingredients. Since lipolytic enzymes are present in several baking adjuncts, knowledge of this activity is essential to the understanding of the influence of lipase in baking and in the storage of baked products. The lipolytic activities for flour and a

TABLE I
MANOMETRIC ASSAY OF BREAD CONSTITUENTS FOR LIPASE ACTIVITY USING
MONO-N-BUTYRIN AS A SUBSTRATE

Source of enzyme	Quantity of enzyme source	CO_2 evolved
	mg.	$\mu\text{l.}$
Patent flour	100	1.4
Malted wheat flour	100	1.4
Low grade flour	100	3.9
Malted barley (ground whole)	100	14.2
Soybean meal (fat extracted)	100	58.8
Wheat germ (fat extracted)	100	132.4
Fungal amylase concentrate	100	141.0
Yeast (compressed)	100	727.0
Yeast (dried)	100	1400.0
Pancreatin (commercial)	100	3150.0
Baked bread (containing 10% defatted wheat germ)	445	00.0

number of possible dough ingredients are listed in Table I. The activity is expressed in $\mu\text{l.}$ of carbon dioxide released from the buffer during 30 minutes at 37°C .

It is striking to note that compressed yeast as employed by the baker contained approximately 500 times as much lipase activity as did

ordinary flour compared on an uncorrected moisture basis. This value which was corrected for respiration was confirmed by means of the titrimetric procedure used by Sullivan and Howe (15). Thus, the 2% yeast normally added in baking would contain approximately 10 times as much fat splitting enzyme activity as the entire quantity of flour used. It would therefore appear that the addition of yeast to premix preparations would be highly undesirable. A few such preparations contain the yeast packaged separately. In consideration of the present data this technic should contribute to the shelf life of the food material.

TABLE II
EFFECT OF SATURATED AND UNSATURATED FATTY ACIDS IN STRAIGHT
DOUGH BAKING PROCEDURE

Cottonseed oil	Fatty acids	Loaf characteristics	
		Loaf volume	Grain and texture
g.	g.	ml.	%
CHECKS			
0.0	0.0	805	80
3.0	0.0	855	80
3.0 ¹	0.0	885	85
SATURATED FATTY ACIDS FROM COTTONSEED OIL			
2.5	0.5	840	88
2.0	1.0	830	88
1.0	2.0	800	88
0.0	3.0	785	86
UNSATURATED FATTY ACIDS FROM COTTONSEED OIL			
2.5	0.5	820	80
2.0	1.0	785	80
1.0	2.0	775	80
0.0	3.0	730	80

¹ Hydrogenated cottonseed oil.

The high lipolytic activity of wheat germ as compared to that for patent flour or even low grade flour corroborates the work of Engel (5) but is not in agreement with the findings of Sullivan and Howe (15) or Pett (10).

As might be expected, the lipase activity present in the normal dough ingredients or that provided by the addition of defatted wheat germ was entirely destroyed in the baking process. Lipase is thus nonfunctional during the storage of baked goods, and hydrolytic ran-

cidity due to enzyme action would not be expected to occur in baked goods such as rations which are normally stored for long periods of time.

The Influence of Free Fatty Acids on Baked Bread. Due to its availability, cottonseed oil rather than wheat fat was used as the source of fatty acids for this study. The same ratio of saturated to unsaturated fatty acids is present in both oils. Data in Table II obtained by the straight dough procedure show that as little as 0.5% of either saturated or unsaturated fatty acids caused marked deterioration in loaf volume. Only the unsaturated acids failed to improve the internal score, however. Similar results were obtained with the sponge procedure.

TABLE III
ASSAY OF WHEAT PRODUCTS, ENZYME PREPARATIONS, AND BREAD
CONSTITUENTS FOR LIPOXIDASE ACTIVITY USING
CAROTENE AS A SUBSTRATE

Source of enzyme	Quantity of enzyme source	Carotene destroyed in one hour
	mg.	%
Commercial flour	450	7.3
Patent flour fraction	450	7.6
Clear flour fraction	450	24.7
Bran fraction	450	20.6
Shorts fraction	450	43.1
Germ fraction (defatted)	100 ¹	32.0
Yeast (compressed)	450	1.4
Malted wheat flour	250	1.6
Vegetable amylase concentrate	26 ²	0.0
Fungal amylase concentrate	31 ²	0.0
Cereal amylase concentrate	87 ²	2.0
Bacterial amylase concentrate	34 ²	0.0
Soybean meal (defatted)	1 ³	67.0
Bread	900 ³	0.0

¹ 450 mg. of the germ fraction (defatted) destroyed 100% of the carotene in less than 30 minutes.

² Equivalent to the alpha-amylase activity in 3 g. of malted wheat flour.

³ Contains 550 mg. flour and 20 mg. defatted soybean meal.

The inferior results obtained when the unsaturated fatty acids were used and the superior results obtained when a partially hydrogenated cottonseed oil was used may be due in part to the difference in physical state of the fatty acids. Baker and Mize (1) reported that bread doughs mixed and proofed below the melting point of coconut oil gave results that corresponded in all respects to those obtained with semi-solid or high viscosity fats. Doughs mixed and proofed above the melting point of coconut oil produced less desirable results similar to those obtained when liquid low-viscosity fats were employed.

Striking deteriorative changes were also evidenced by consumer acceptability ratings. A six member panel registered marked un-

favorable reactions and reported a lingering tallowy taste for bread baked with as little as 0.5% free fatty acid of either type. Sinclair and McCalla (12) found that the amount of free acid liberated in one sample of flour, which had been stored in a sealed can for 16 months, was equal to 1% of the flour. Bread baked from such flour would be expected to be nonacceptable.

The Lipoxidase Activity of Baking Ingredients. The presence of lipoxidase in wheat germ has been demonstrated by Sumner (17); however, no data are available concerning the amount of this enzyme in other wheat fractions or in the various possible baking adjuncts. A summary of the lipoxidase activity of a series of wheat fractions and amylolytic preparations is given in Table III. Of the materials investigated only wheat germ and soybean meal contained a large quantity of this enzyme. The small quantities of lipoxidase present in flour, however, may be sufficient to cause significant deteriorative changes if storage is extended for a long time under unfavorable conditions.

The lipoxidase activity of normal bread ingredients plus that present in an added 10% of soybean meal was completely inactivated during the baking process. Thus, this enzyme does not function during storage of baked goods and it may be concluded that any oxidation occurring during storage of baked products is nonenzymatic in nature.

The Influence of Oxidized Fats on the Quality of Baked Bread. The products obtained by air oxidation of the melted shortening were found to have a negligible effect on the physical characteristics of baked bread. COVO shortening with peroxide values ranging from 0 to 62.0 and hydrogenated cottonseed oil possessing peroxide values as high as 48.0 produced no noticeable changes in either loaf volume or grain and texture. A marked difference in flavor and aroma sensations was found, however, between the unoxidized samples and those with a slight amount of oxidation (peroxide number of 5). It was difficult to distinguish between, and especially to assign numerical scores to, bread baked with oxidized shortenings which possessed peroxide numbers varying between 5 and 62. Some of the difficulties encountered in judging consumer preference might have been eliminated had a ranking technic been used in which the rating given a certain sample depends on the other samples in the test (Bliss, Anderson, and Maryland, 4). This procedure is adaptable either to untrained groups or to experienced judges.

The fact that the oxidized lipids were in the form of triglycerides may explain the difference in these results as compared to those ob-

tained by Sullivan *et al.* (16). While these workers (16) reported that the injurious effect of unsaturated fatty acids was much more pronounced when they became oxidized, it would be expected that the organoleptic reactions would be similar for oxidized unsaturated fatty acids and for oxidized triglycerides.

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SUNFLOWER SEED PROTEIN¹

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ABSTRACT

The isolation of sunflower seed protein has been investigated to determine its probable value for industrial uses. The principal method of protein isolation was by alkali extraction and acid precipitation, and the protein isolated by this method was compared in color and yield with that obtained by salt extraction. The chlorogenic acid in the meal retarded the dispersion of the protein. Due to the presence of chlorogenic acid, the added alkali imparted a green color to the meal and to the isolated protein. Hot 70% ethanol or near absolute methanol were used to remove the chlorogenic acid, but they severely denatured the protein. The alkali-extracted protein had a nitrogen value of about 16.4%, and protein prepared from methanol-extracted meal followed by salt extraction and dialysis had a nitrogen value of 18.69%.

The continuous search for new oilseeds to help supply the oil and fat requirement of the United States has focused considerable attention on sunflowers. The extracted oil of the sunflower seed is without appreciable color and is reported to have good stability. The dehulled seeds have a pleasant sweetish taste and several nutritional investigators (2, 4, 5) show the oil-free meal to have a high nutritional value.

Milner, Hubbard, and Wiele (6) have examined the chemical composition and oil content of 28 samples, comprising four varieties of sunflower seed. Their results for the whole seed show a range of protein content ($N \times 6.25$) for the four varieties of 18.04–21.40% and of 27.47–30.78% for the oil content.

The hulls constitute 39–46% of the seed for the varieties studied. When the hulls are removed the range of protein values is 29.4–32.3% and of the oil content 46.6–53.2%. The dehulled and oil-free meal will have a protein content ($N \times 5.4$) of 52.0–65% as shown in Table I. We have used the nitrogen factor of 5.4 here since it is more consistent with our data for the nitrogen content of the purified protein (see Table II).

The latest publication on isolation of sunflower seed protein is that of Osborne and Campbell (7) in 1897. Earlier studies were made by Ritthausen (8) and Vines (12).

Osborne and Campbell prepared a series of 10 protein fractions from one batch of oil-free meal by salt extraction methods and found

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TABLE I
SOLVENT-EXTRACTED SUNFLOWER SEED MEALS

Solvent used	Moisture ¹	Ash	Nitro- gen	Protein (N \times 5.4)	Oil	Sugar
Pet. ether (b.p. 30°-60°C.)	9.46	8.42	9.68	52.3	—	—
Ethanol—at room temperature	7.47	5.32	7.98	43.1	25.83	—
Ethanol ¹	10.20	7.35	10.92	59.0	1.29	—
Pet. ether followed by ab. methanol ¹	9.60	9.09	11.88	64.1	—	—
Pet. ether followed by 70% eth- anol ¹	11.62	9.19	11.62	62.7	—	—
ORIGINAL SUNFLOWER SEED SAMPLE						
Whole sunflower seed	—	3.45	3.42	18.47	30.78	3.79
Hulled sunflower seed	—	4.07	5.12	27.65	48.80	6.14

¹ Temperature of alcoholic extractions 60°-70°C.

them to have essentially the same nitrogen content, that is, with a range of 18.00-18.24%. They concluded that, "the most abundant protein of sunflower seed consists of a single globulin." Their first preparations were dark in color, owing to the presence of a substance known at that time as helianthotannic acid. In more recent studies, Gorter (3) identified this compound as chlorogenic acid. To remove the chlorogenic acid, Osborne and Campbell extracted the meal with ethanol of 0.820 specific gravity at 65°-75°C. and obtained a protein containing 18.58% nitrogen. He states that the last preparation "was freer from coloring matter than any before made," but he believed that he had not succeeded in separating it completely.

In view of possible expansion in the production of sunflowers as a farm crop it was decided to study isolation of the protein by the method of alkali extraction and acid precipitation, and to examine the isolated protein for industrial utilization. The sunflower seed used in this study was the Sunrise type.

Materials and Methods

The analyses of the solvent-extracted proteinaceous materials used for the nitrogen dispersion experiments and for protein isolation are shown in Table I, which also includes analysis of the original seed. The oil-extracting solvents were (a) petroleum ether, b.p. 30°-60°C., (b) 95% ethanol, (c) petroleum ether followed by 70% ethanol, and (d) petroleum ether followed by absolute methanol. The oil extractions were carried out in a modified Soxhlet extractor. For the hot alcohol extractions the temperature was 60°-70°C., and for the cold ethanol the temperature range was 25°-35°C.

The elapsed time for the hot alcohol extractions was approximately 16 hours and for the cold ethanol extraction, 60 hours.

The dispersion of the nitrogen compounds from the oil-free meal at various pH values was carried out by a procedure described in detail in an earlier publication by Smith and Circle (10) for studies on soybean protein. Two and a half grams of the finely ground oil-free meal and 100 ml. of the dispersing solution were placed in a 250 ml. centrifuge bottle and shaken mechanically for 30 minutes. The undispersed portion was removed in a centrifuge developing a maximum relative centrifugal force at the bottle tip of 1,975 times gravity and an aliquot of the dispersion taken for nitrogen analyses. The hydrogen-ion concentrations were determined with a glass electrode pH meter. For the precipitation experiments and protein isolation the meal was extracted in the pH range of 11.0-12.5. Sulfuric acid was used to lower the pH in the precipitation experiments; however, hydrochloric acid was used for protein isolation procedures. The dispersion and precipitation data and protein yields are based on the total nitrogen in the dehulled and solvent-extracted meal.

The nitrogen dispersion and precipitation data at various pH values for dehulled, petroleum ether extracted sunflower seed are shown in Fig. 1. In the precipitation data the meal was extracted at a meal-to-

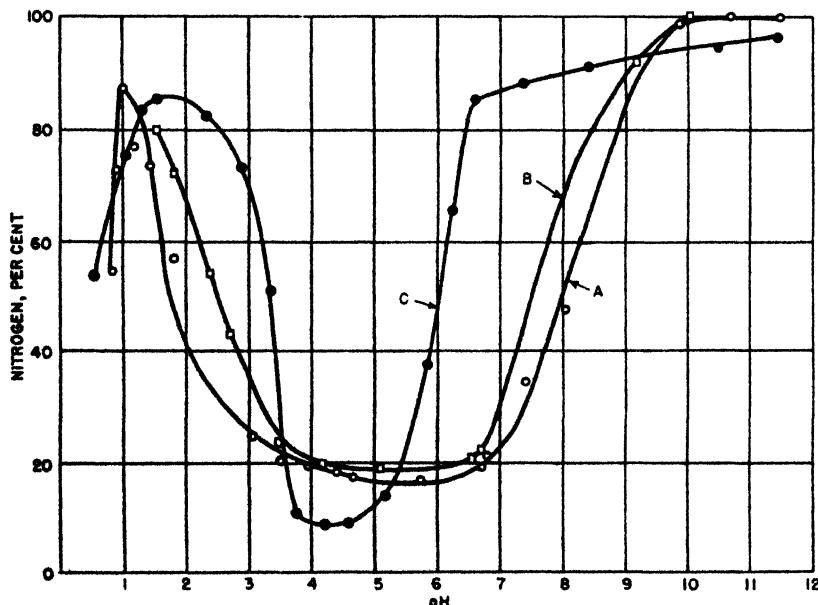


Fig. 1. Nitrogen-pH dispersion and precipitation curves for dehulled, petroleum ether extracted sunflower seed meal. Curve A, nitrogen-dispersion curve; B, nitrogen-precipitation curve; C, nitrogen-dispersion curve for soybean meal included for comparative purposes.

solvent ratio of 1:40 and at pH of about 11.0, and the undispersed residue removed in the centrifuge. The protein was precipitated from aliquots of the alkaline extract by addition of varying amounts of sulfuric acid. The precipitate was removed in the centrifuge and the difference between the percentage of nitrogen originally present in the dispersion and that left after removal of the precipitate gave a measure of the amount of protein in the precipitate. For comparative purposes, Fig. 1 also shows a pH-nitrogen dispersion curve for soybean meal taken from Smith and Circle (10).

Results

In comparing the data for the sunflower meal with soybean meal, the sunflower has a very broad minimum in the pH range of 3.0-7.0 for both the dispersion and precipitation curves. This same relationship is true also in comparing the sunflower meal curve with curves previously published for vegetable proteins such as peanut (1) and flax (11).

The water extract of the sunflower seed meal has a pH value of 6.6 but disperses only 20% of the total nitrogen. This is the same pH value as the water extracts from freshly flaked, solvent-extracted soybean, peanut, and flax meals, but the latter extracts contain 85-95% of the total nitrogen of the meals. The water extract of the sunflower seed meal contains very little protein which can be precipitated either with acid or by heating, whereas a high percentage of the protein in the other extracts is precipitated by adding acid. However, the nitrogen in the sunflower seed meal is completely dispersed at pH 10.0.

The broad minimum in the dispersion curve of the sunflower seed meal and the low water dispersibility of its protein might be attributed to the presence of chlorogenic acid, a tannin-like compound widely distributed in plant life. The dispersibility of the protein-chlorogenic acid complex at pH 10.0 is consistent with the negative reaction of vegetable tannins with proteins in the alkaline region and suggests that the complex formed between the protein and chlorogenic acid is easily dissociated.

In Fig. 2 are the nitrogen-pH dispersion curves for the sunflower seed meal which has been solvent extracted with hot 95% ethanol, for that extracted with cold ethanol, and including the protein precipitation curve of the protein from the hot ethanol extraction. The cold ethanol was such a poor oil solvent that it removed only about half of the oil.

A comparison of the curves in Fig. 2 with one another and with those in Fig. 1 shows that after extraction with 95% ethanol, either hot or cold, nitrogen solubility between pH values 3.0 and 7.0 was

lowered as much as 9.0%, and at pH 10.0 or higher the nitrogen dispersibility was lowered from 100% to 95%. The lowering of the dispersion curve between pH 3.0 and 7.0 may be explained as due to loss of soluble nitrogen compounds in the ethanol, or to partial denaturation of the protein and consequent loss in dispersibility, or both. Since there is such slight difference between the curves for the cold and hot ethanol extractions, the effect of denaturation on dispersibility is thought to be rather small. This is further substantiated by the comparatively small shift in the curve to a higher pH range

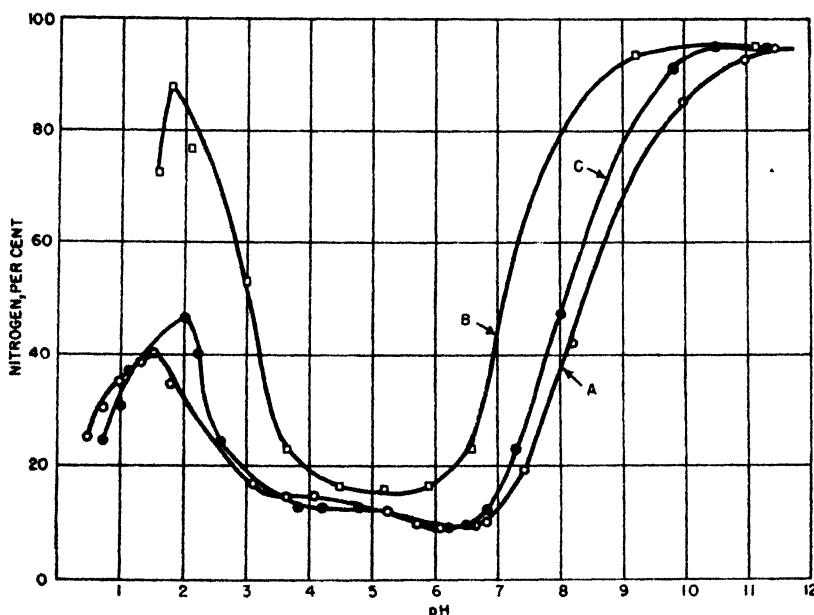


Fig. 2. Nitrogen-pH dispersion and precipitation curves for dehulled, ethanol-extracted meal. Curve A, nitrogen-dispersion curve for hot-ethanol-extracted meal; B, nitrogen-precipitation curve for protein from hot-ethanol-extracted meal; C, nitrogen-dispersion curve for cold-ethanol-extracted meal.

which would be expected to result from denaturation. The 95% ethanol did not remove an appreciable amount of the chlorogenic acid. This was shown by the color of protein isolated from the alcohol-extracted meal, which was as dark in color as that obtained from the hexane meal.

Another indication of the presence of chlorogenic acid was the bright chrome yellow color which was obtained by adding sodium hydroxide to the meal. The yellow color changed to green on air oxidation or by chemical oxidation. The rate of oxidation or color change varied with pH of the dispersion; at pH 9.0 the green color appeared in 8-10 minutes. At pH 11.5 or higher, the color changed

directly to brown. The appearance of the green or brown color can be prevented by the use of reducing agents such as dithionite salts, but the color will partly return on washing out the reducing agent and exposing the protein to the air.

In further attempts to remove the chlorogenic acid, the petroleum ether extracted meal was re-extracted with acetone, carbon tetrachloride, benzyl alcohol, and benzene but without success.

Seventy per cent ethanol and near absolute methanol removed the chlorogenic acid from the meal in about 3 hours in a Butt extractor,

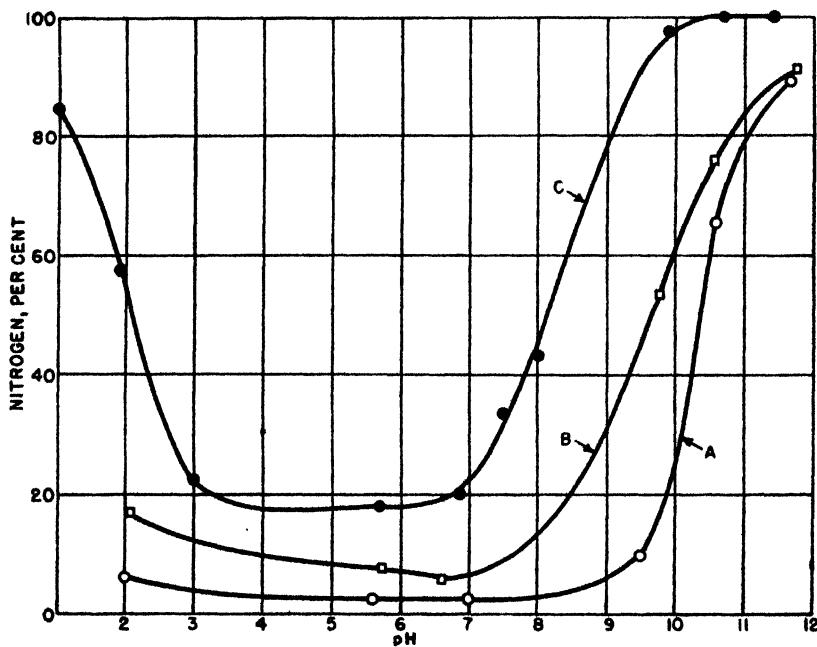


Fig. 3. Nitrogen-pH dispersion curves. Curve A, for meal extracted with hot 70% ethanol; B, for hot-near-absolute methanol; C, from petroleum ether extracted flakes included for comparative purposes.

and 70% isopropanol in about 5 hours. These extractions were made on meal which had been freed from oil with petroleum ether. Concentrations of the alcohols above and below 70% did not show any improvement in results.

The protein prepared from the above doubly extracted meals was light in color. While it was quite free of chlorogenic acid as determined by the alkali test, the protein was severely denatured as demonstrated by the pH-nitrogen dispersion curves shown in Fig. 3, where curve A is for the 70% ethanol-extracted meal, B for near absolute methanol, and C for hexane meal which was included for com-

parison. The 70% ethanol had a greater adverse effect on nitrogen dispersion than the methanol; this relationship would be expected due to the effect of the water.

Discussion

Proteins were isolated from the sunflower seed meals which had received various solvent extraction treatments in order to estimate the extent of chlorogenic acid removal by comparing the color of the isolated proteins and to determine the relative purity of the proteins by nitrogen analysis.

In the following described methods of protein isolation, samples A, B, C, and D were from hexane-extracted meals and E was from hexane-extracted meal followed by methanol extraction.

- A. Petroleum ether extracted meal—Alkali extracted twice at ratios of 15:1 and 8:1 and acid precipitated; redispersed in alkali and reprecipitated once; washed twice with water.
- B. Petroleum ether meal—Alkali extracted twice at ratios of 15:1 and 8:1 and acid precipitated; redispersed in alkali and reprecipitated twice; washed twice with water after each precipitation.
- C. Petroleum ether meal—Alkali extracted twice at ratios of 15:1 and 8:1 and acid precipitated; the curd washed twice with water and four times with boiling 70% ethanol.
- D.^o Petroleum ether meal—Extracted with 10% salt solution at ratio of 12:1 and dialyzed 7 days.
- E. Petroleum ether meal followed by methanol extraction—Extracted with 10% salt solution at ratio of 12:1 and dialyzed 4 days.

The results of the protein analysis in Table II show that the alkali-extracted and acid-precipitated proteins A and B are lowest in nitrogen

TABLE II
ANALYSIS OF ISOLATED PROTEINS

Sample ¹	Moisture	Ash	Nitrogen	Nitrogen moisture ash free	Yield ²	Color
A	9.22	0.28	14.89	16.45	32	Dark green
B	8.94	.48	14.84	16.39	31.6	Dark green
C	9.99	1.44	15.61	17.63	31.8	Light green
D	9.32	.18	15.76	17.42	22.0	Light brown
E	0	1.82	18.35	18.69	4.6	Very light brown

¹ See text for description of protein preparations.

² Based on weight of the oil-free meal.

content and that redispersion, reprecipitation, and water washing do not increase the purity of the protein. The color of proteins A and B is dark green. Washing the protein curd with 70% ethanol is quite effective in removing the chlorogenic acid as illustrated by sample C, although the light green color of this protein shows incomplete removal of the chlorogenic acid with this amount of alcohol washing. Protein prepared by salt extraction and dialysis of the petroleum ether meal, sample D, is quite impure in comparison to sample E, which was prepared from petroleum ether extracted meal followed by methanol extraction. Protein D was light brown. Sample E was lightest in color of all the proteins and showed no chlorogenic acid by the alkali test.

The dark color of the protein prepared from the petroleum ether and the 95% ethanol-extracted meal would exclude its general acceptance as an industrial protein. Although 70% ethanol and methanol were effective solvents for the removal of chlorogenic acid, their use would increase considerably the difficulty of protein isolation.

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MINERAL ANALYSIS OF PERUVIAN WHEAT¹

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ABSTRACT

Wheat introduced into Peru during the period of the Spanish conquest has been grown predominantly on the same terraced mountain farms since that time. Under the circumstances it might be expected that the wheat would differ somewhat from that grown on the Great Plains Area of the United States.

Forty-six samples of wheat from various locations in Peru were evaluated for protein, ash, gluten quality, and baking quality, and the ash analyzed quantitatively for the following eight elements: potassium, phosphorus, magnesium, calcium, sodium, manganese, iron, and copper.

The total mineral content of Peruvian wheats was found to be within the limits usually reported for North American wheats; however the phosphorus, manganese, and magnesium content of the ash was lower than usually reported. Within the group of samples, only the elements potassium, iron, and copper were significantly correlated with the total ash content.

The protein content and baking quality of the samples varied widely. There was no significant correlation between total ash and protein content.

The wheat plant was not indigenous to either the North or South American continents. Wheat is reported to have been introduced first into Mexico in 1530 when one of Cortez's slaves found several wheat grains which had accidentally been mixed with some rice (5). It is probable that subsequent wheat development in Mexico and Central and South America came from this source.

Wheat appeared in Peru along with the Spanish conquest several years later. Since Pizarro found agriculture highly organized in the Inca Empire, it is probable that wheat was readily introduced into the agricultural system and has been grown on many of the same terraced areas of the Andes for the past 400 years. It is therefore of interest to compare the mineral constituents of wheat from Peru with wheats produced on newer soils and under radically different environmental conditions.

The mineral analysis of wheat ash has attracted much attention, and several excellent reviews of the literature have been given by Sullivan (9), Beeson (2), and Bailey (1).

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Contribution No. 149, Department of Milling Industry and No. 362, Department of Chemistry,
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TABLE I
DESCRIPTION AND ANALYSIS OF PERUVIAN WHEATS

Sample no.	Location where grown	Type of wheat	Test weight	Protein ¹	Ash ¹	Loaf volume
1	Celendin	White vulgare	58.5	12.1	1.70	790
2	Huanuco	White vulgare	57.9	13.7	1.69	835
3	Huanuco	White vulgare	54.6	9.5	2.56	545
4	Huanuco	Red vulgare	58.5	12.1	2.17	590
5	Huanuco	White vulgare	57.7	13.7	1.57	775
6	Huanuco	White and red vulgare	55.6	9.1	1.53	625
7	Huacho	White vulgare	59.5	11.8	1.60	700
8	Tarma	Amber durum	55.2	9.1	2.22	450
9	Concepcion	White vulgare, 5% durum	59.5	7.6	1.53	340
10	Concepcion	Red and white vulgare	57.8	9.4	1.60	350
11	Concepcion	Red and white vulgare	57.3	10.7	1.36	495
12	Cajamarca	Red vulgare	60.0	12.1	1.49	880
13	Cajamarca	White vulgare	57.9	7.6	1.70	470
15 ^a	Cajamarca	White vulgare	59.8	7.8	1.68	445
16	Cajamarca	White vulgare	60.5	7.8	1.45	485
17	Cajamarca	Red vulgare	57.5	9.4	1.78	595
18	Cajamarca	White vulgare	57.5	11.3	1.62	600
19	Cajamarca	Red vulgare	61.2	9.1	1.65	440
20	Cajamarca	White vulgare	58.4	11.6	1.48	705
21	Cajamarca	Red, few white vulgare	58.2	10.4	1.29	740
22	Cajamarca	White poulard	56.2	7.9	1.31	440
23	Huancayo	White vulgare	57.6	8.5	1.48	455
24	Huancayo	White vulgare	59.4	8.0	1.35	325
25	Huancayo	Amber durum	58.2	9.1	2.14	310
26	Huancavelica	White vulgare	54.3	9.6	1.46	400
27	Cusco	White vulgare	55.9	6.3	1.77	440
28	Cusco	Red vulgare	59.2	7.9	1.41	560
29	Cusco	White and red vulgare	57.4	8.6	2.07	540
30	Cusco	White and red vulgare	59.5	10.4	1.48	535
31	Cusco	White and red vulgare	56.1	8.6	1.71	595
32	Cusco	White and red vulgare	56.4	8.9	1.54	505
33	Camana	Amber durum	57.1	10.9	2.13	505
34	Arequipa	White vulgare	58.6	11.0	1.62	585
35	Arequipa	Red and white durum	56.0	11.2	1.69	475
36	Arequipa	White vulgare	61.4	11.6	1.44	740
37	Arequipa	Hard white vulgare	60.2	12.1	1.32	750
38	Arequipa	Red vulgare	58.6	14.1	1.67	835
39	Arequipa	Amber durum	58.2	11.9	1.55	585
40	Arequipa	White vulgare	56.0	11.2	1.69	770
41	Moquequa	White vulgare	60.4	9.7	1.84	675
42	Moquequa	White vulgare	62.2	8.6	1.57	705
43	Moquequa	Amber and red durum	56.8	8.7	1.90	530
44	Moquequa	Red vulgare	58.2	11.9	1.95	735
45	Tacna	White vulgare	59.3	9.7	1.73	580
46	Tacna	Red and white vulgare	59.2	9.7	1.73	660
47	Tacna	White vulgare	48.6	9.5	1.77	605

¹ Results reported on 14% moisture basis.

^a No. 14 was hull-less barley.

Materials and Methods

Forty-six samples of wheat were obtained from different locations in Peru (8). The location from which the samples came, the type of wheat, the protein, ash, and test weight are recorded in Table I.

The wheat samples were ground, mixed, and ashed by usual procedures. Ashing was done at 600°C. The ash was then analyzed for potassium, phosphorus, magnesium, calcium, sodium, manganese, iron, and copper. All elements except potassium and calcium were determined spectrographically. All data are reported on a dry weight basis.

Potassium was determined by a method recommended by Harris (3). A procedure suggested by Wang (11) for calcium in blood serum was modified slightly for the calcium determination. A 20 mg. sample of ash was dissolved in the buffer solution, after which the procedure of Wang was followed.

The remaining mineral constituents were determined spectrographically on a Bausch and Lomb large litrow spectrograph, using the standard solutions, spectral lines, and techniques recommended by Morris *et al.* (6) with the following modifications.

Samples were placed in solution and on electrodes as recommended by Morris (6). The image was focused on the collimating lens and electrode spacing was set at one mm. Samples were arced five minutes. The excitation source was a high voltage A.C., operating at 2,200 volts and 2.4 amperes. The sector was set at three-eighths open. All spectra were taken in duplicate and a set of standards was placed on each plate. Line densities were read with the aid of an ARL—Dietert densitometer. This is essentially the same technique as used in the determination of the mineral content of Kansas wheat (7).

Results and Discussion

The data recorded in Table I show that the samples were obtained from representative wheat growing areas throughout Peru from Celen-din in the North to Tacna near the Chilean boundary. Most of the samples were white or red vulgare types, but durum and one sample of white poulard are included. The protein, ash, and test weight are also included in Table I. The protein content varied over a wide range from 14.0 to 6.3%, and the ash content varied from 2.56 to 1.29%. There was no significant correlation between protein and total ash content. This is in contrast to the significant relationship between protein and mineral content reported by Schrenk and King (7) for Kansas grown wheats.

The average analysis of the ash of the 46 samples for the eight elements, potassium, phosphorus, magnesium, calcium, sodium, man-

TABLE II
COMPARISON OF THE AVERAGE MINERAL ANALYSES OF DIFFERENT WHEATS

Source	Protein	Ash	K	P	Mg	Ca	Na	Mn $\times 10^3$	Fe $\times 10^3$	Cu $\times 10^3$
Peru	%	%	%	%	%	%	%	0.0062	0.17	0.39
Kansas ¹	11.9	1.94	0.40	0.14	0.042	0.059	0.117	0.0170	0.48	0.06
Marquis ²	14.8	2.07	0.40	0.43	0.135	0.046	0.0170	0.0030	0.24	0.64
Tennarq ³	15.9	2.05	0.52	0.44	0.190	0.045	0.0043	0.0043	0.42	0.06
Trumbull ⁴	14.9	1.79	0.37	0.53	0.099	0.046	0.027	0.0085	0.57	0.44
	11.3	1.99	0.47	0.53	0.134				0.65	0.10

Results reported on moisture-free basis.

¹ Data of Schrenk and King (7).

² Data of Sullivan and Near (10) and Howe and Sullivan (4).

³ Data of Morris, Pascoe, and Alexander (6).

TABLE III
COMPARISON OF THE MEAN ASH CONTENT AND MINERAL ANALYSIS, INCLUDING STANDARD DEVIATIONS OF PERUVIAN AND KANSAS WHEATS

Source	Total ash		Potassium		Phosphorus		Magnesium		Calcium		Sodium		Manganese		Iron		Copper	
	M ¹	S.D. ²	M	S.D.	M	S.D.	M	S.D.	M	S.D.	M	S.D.	M	S.D.	M	S.D.	M	S.D.
Peru	1.94	.3155	.37	.0666	.136	.0377	.042	.0249	.059	.0164	.0062	.0026	.0017	.0003	.0042	.0039	.0042	.0012
Kansas	2.07	.2152	.40	.0354	.43	.0691	.135	.0154	.046	.0091	.0170	.0027	.0048	.1548	.0074	.1927	.0067	.0963

Results reported on moisture-free basis.

¹ M = mean.

² S.D. = standard deviation.

ganese, iron, and copper, is reported in Table II. In Table II the mineral analyses of Peruvian wheat are compared with similar data reported by Sullivan and Near (11), and Howe and Sullivan (4) for Marquis type spring wheat; the data of Morris, Pascoe, and Alexander (6) for the two red winter varieties, Tenmarq and Trumbull; and the extensive data reported by Schrenk and King (7) for hard red winter wheat. The standard deviations in the mineral analysis of the Peruvian as compared with the Kansas wheat are reported in Table III.

The total mineral content of the Peruvian wheats was found to be within the limits usually reported for North American wheats, even though great differences in variety, climate, and soil exist. The standard deviations of the total ash of the Peruvian samples compared with the analysis of wheat grown in Kansas were 0.3155 and 0.2052, respectively. Considering the wide geographical area from which the samples were obtained, the differences in type of wheat and different agricultural practices, the amount of variability in mineral matter in the Peruvian samples is not excessive.

Comparing the average values of the eight elements with similar results of other wheats, it is evident that the phosphorus, manganese, and magnesium content of the ash of Peruvian wheats is somewhat lower. In Table III the standard deviations of the elements in the ash of Peruvian wheats are compared with Kansas wheat. The magnesium, calcium, and potassium variability was greater among the Peruvian samples, but the elements iron, copper, phosphorus, and manganese showed less variability than the Kansas samples.

The degree of association between the different elements and the total ash for the Peruvian and Kansas wheats is compared in Table

TABLE IV
COMPARISON OF THE CORRELATION COEFFICIENTS BETWEEN THE TOTAL ASH CONTENT
AND SEVERAL INDIVIDUAL ELEMENTS IN THE ASH OF
PERUVIAN AND KANSAS WHEATS

Variables	Correlation coefficients	
	Peruvian	Kansas
T.A.K	0.37**	0.62*
T.A.P	0.11	0.68**
T.A.Mg	0.12	0.76**
T.A.Ca	-0.06	0.61*
T.A.Na	0.00	0.86***
T.A.Mn	0.14	0.46
T.A.Fe	0.68***	0.74**
T.A.Cu	0.38**	0.61*

A = Total ash.

* = Slightly significant.

** = Significant.

*** = Highly significant.

IV. Among the Peruvian samples only the elements iron, copper, and potassium were significantly correlated with the total ash content. In contrast, among the Kansas samples all the elements were significantly correlated with the total ash content except manganese. Data of Bailey and Hutchinson reported by Bailey (1) gave the following correlation coefficient between magnesium, calcium, iron, and copper content and total ash for spring wheat: $r_{A.Mg} = 0.75$; $r_{A.Ca} = 0.29$; $r_{A.Fe} = 0.55$, and $r_{A.Cu} = 0.12$. It is thus apparent that considerable variation occurs in the relationship between total ash and the content of the various elements present.

The total ash content and mineral analysis of Peruvian wheat compared favorably with similar results obtained from North American wheats. There was no evidence that three hundred years of wheat production in the same locations, under Peruvian conditions, had caused notable alterations in these common characteristics of wheat.

Acknowledgments

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THE DETERMINATION AND DEFINITION OF COLOR IN EGGS¹

GASTON DALBY²

ABSTRACT

A method for the extraction of egg color by acetone, the comparison of this extract with an acidified potassium dichromate standard, and a method of reporting color in terms of a color index which is 25 times the number of milligrams of potassium dichromate equivalent to the color extracted from 1 g. of egg, has been recommended by the Committee. An acceptable commercial mixed egg will have an index of 100. The color of eggs in terms of the pigments present is discussed. Transmission curves of pure pigments, compared to egg extracts and to an acidified potassium dichromate standard are shown.

The color of eggs is of commercial importance to bakers and food manufacturers who use eggs in their products. The consumer tends to judge richness of the product in accordance with the apparent amount of egg color it contains. It is not known if there is a difference in nutritional value between a light and good colored egg—the analogy to winter and summer butter may, however, suggest that there is. The value of good colored eggs to the food processor, therefore, is that of attractiveness and enhanced consumer appeal.

It is relatively common practice in food laboratories to extract the color from eggs with acetone, butyl alcohol, or other solvents, and compare the color of the extract with some standard. Turner and Conquest (3) published such a procedure. Munsey (2) in his study of pigments in egg noodles also suggested a similar type of method. One purpose of the Committee was to study the various available procedures and to recommend a standard one to the Association.

After the egg color is extracted, the next problem is to find a suitable standard with which to compare the extract. Potassium dichromate generally has been used for this purpose. Standard reproducible solutions can easily be made with this reagent, and in color it is a close match to the average pigments extracted from eggs. After the colored extract is compared with the dichromate standard the question arises as to how the color of the eggs shall be reported. Shall this be in terms of dichromate concentration, in terms of Tintometer filter factors, or percentage transmission at a specified wave length?

¹ Manuscript received June 2, 1948.

² Report of the Committee on Methods for the Determination and Definition of Color in Eggs.

² Ward Baking Company, New York, N. Y.

The recommendation of a suitable color standard and a method of reporting egg color were also objectives of the Committee.

Materials and Methods

Apparatus: (1) Spectrophotometer, photoelectric colorimeter, Duboscq or similar visual colorimeter, Nessler tubes, or comparator tubes. (2) Filter papers: fluted. Whatman No. 12.

Reagents: (1) Acetone: technical grade or better. (2) Ammonium hydroxide: 15N. (3) Potassium dichromate soln.: 0.020% in 1.5 M ortho-phosphoric acid. Prepare by dissolving 0.200 g. potassium dichromate (Reagent Grade) and 102 ml. 85% phosphoric acid in water and diluting to 1 liter with water. This solution contains 0.2 mg. potassium dichromate per ml.

Weigh 5 g. of mixed eggs, or 2 g. of yolk, into a tared weighing dish. Add 5 ml. of water and mix thoroughly with a stirring rod. Transfer to a 100 ml. volumetric flask, but do not attempt at this time to remove the sample which adheres to the weighing dish. Add *immediately* about 50 ml. of acetone to the flask; stopper, and shake. Wash weighing dish with acetone and add washings to the volumetric flask. In some cases, the addition of a few drops of 15N ammonia water will be helpful in the prevention of a cloudy solution. Make up to volume with acetone and shake. After a few minutes standing, filter through Whatman No. 12 folded filter paper, and compare clear filtrate with standard solution.

Color Standard—Visual Colorimeters. Use a 0.020% solution of a reagent grade potassium dichromate in 1.5 molar phosphoric acid.

Spectrophotometers and Electric Colorimeters. Prepare standard curve with 0.010%, 0.020%, and 0.030% potassium dichromate in 1.5 molar phosphoric acid as may be required for the particular instrument available.

Nessler and Comparator Tubes. With these use more dilute standards and extract. Prepare the extract of 5 g. of mixed egg made up to 100 ml. Make a second dilution of 20 ml. of this extract to 100 ml. with acetone. Compare these extracts against standards containing 0.001, 0.002, 0.003, and 0.004% dichromate in 1.5 molar phosphoric acid.

Method of Reporting Egg Color. From comparison with the standard, the number of milligrams potassium dichromate equivalent to the color extracted from 1 g. of egg product is determined. This number of milligrams of potassium dichromate is then multiplied by the factor "25" to convert to "color index."

Collaborative Study. Two samples of frozen eggs were sent out to each committee member. The results of the collaborative study are shown in Table I.

Discussion

(a) *Dichromate Standard.* The color shade of the dichromate standard varies according to the ratio of dichromate and chromate ions present. The addition of phosphoric acid to standardize this ratio has been made in accordance with the suggestions of Kitson and Mellon (1). The absorption peak at $450 \text{ m}\mu$ makes it possible to use this inorganic material in 1.5 *M* phosphoric acid solution as a standard for the measurement of organic egg pigments in acetone since the absorption peak of the egg pigments is also at this same wave length. Transmission curves of this acidified dichromate standard, collaborative Samples A and B, Xanthophyll, and Beta Carotene are shown in Fig. 1.

TABLE I
COLLABORATIVE RESULTS OF EGG COLOR DETERMINATION¹

Collaborator	Sample A	Sample B	Type of instrument
1	4.1	6.7	Duboscq
2	5.9	9.0	Duboscq
2	5.4	8.8	Spectrophotometer
3	5.0	8.1	Duboscq
4	4.8	7.4	Nessler tubes
5	5.0	8.0	Nessler tubes
6	5.4	8.8	Electric colorimeter
7	5.0	8.0	Comparator tubes
8	4.0	7.0	Klett
9	5.0	9.3	Duboscq
10	5.3	9.1	Duboscq
11	5.6	9.1	Duboscq
12	4.0	5.0	Nessler tubes
13	5.0	7.5	Nessler tubes
14	2.5 ²	3.0 ²	Comparator tubes
Average Color Index	5.00	8.0	
	125	200	

¹ In mg. dichromate equivalent per gram of egg.

² Not included in the average.

(b) *Method of Reporting Color.* For strictly scientific purposes the color of eggs in terms of milligrams potassium dichromate equivalent per gram of egg is sufficient. Since, however, in commercial practice, egg color values are used in specifications for egg purchases and the values are thus handled by nontechnical sales and purchasing departments, the Committee recommends that the factor "25" be used. Thus a mixed egg with a color equivalent of 4 mg. potassium dichromate per gram will have a color index of 100. The Committee agrees that a mixed egg with a color index of 100 is a good colored product and that this level of color is fair and reasonable to both producer and processor. A mixed egg fortified with yolk may have an index of 200. Thus a production man can readily understand that such a fortified egg

will give twice the color in the finished product as a mixed egg with an index of 100.

(c) *Egg Pigments.* There are two principal pigments present in eggs, carotene and xanthophyll, but these are so nearly identical in color that the difference between them can only be detected by means of a sensitive spectrophotometer. The nature of the transmission curves of these pure pigments, as shown in Fig. 1, demonstrates that egg color is

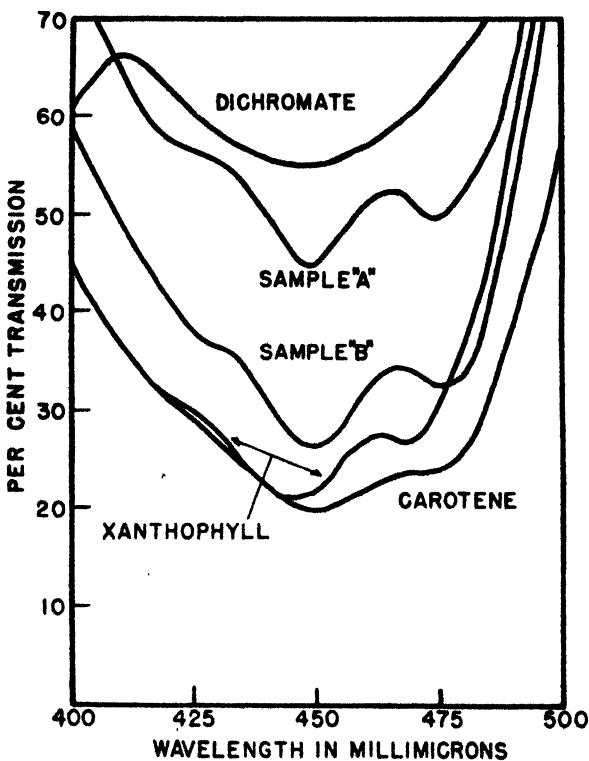


Fig. 1. Transmission curves of collaborative samples A and B, 5 g. to 100 ml. acetone; potassium dichromate, 0.02% in 1.5 molar phosphoric acid; and Xanthophyll and Beta Carotene, 4 mg. to 1 liter of acetone.

not determined by the ratio of "red" to "yellow" pigments as is so commonly assumed, but by the total concentrations of the two pigments which are almost identical in shade of color.

For many years egg color has been evaluated by many laboratories in terms of Lovibond filters. The color has been reported in terms of "yellow" and "red" units. This perhaps is the basis for the assumption that eggs contain "red" and "yellow" factors. The acetone extracts of highly colored eggs appear more red than extracts of light

colored eggs, not because of the presence of increased quantities of a red pigment, but because the higher concentrations of carotene and xanthophyll absorb more of the blue and green components of the incident light, and leave a higher proportion of red in the transmitted light. The color of neutral or acid solutions of potassium dichromate appears more red, with increasing concentrations, for the same reason.

Spectrophotometers and electric colorimeters give most satisfactory results. Visual colorimeters are next in desirability. The least desirable types of apparatus are Nessler tubes and comparator tubes.

Acknowledgments

The chairman wishes to express his gratitude to the following committee members who collaborated in this study: William B. Bradley, American Institute of Baking, Chicago, Ill.; Geo. T. Carlin, Swift and Co., Chicago, Ill.; W. H. Cathcart, The Great Atlantic and Pacific Tea Co., New York, N. Y.; D. B. Davis, Continental Baking Co., New York, N. Y.; Geo. Garnatz, The Kroger Co., Cincinnati, Ohio; R. W. Mitchell, Purity Baking Co., Chicago, Ill.; R. A. Pouchain, Tasty Baking Co., Philadelphia, Pa.; Roland Selman, C. J. Patterson Co., Kansas City, Mo.; Oscar Skovholt, Quality Bakers of America, New York, N. Y.; H. K. Steele, Fleischmann Labs., New York, N. Y.; W. H. Tonkin, Standard Brands, Inc., Terre Haute, Ind.; and N. H. Walker, Arnold Bakers, Inc., Port Chester, N. Y.

The chairman also wishes to express his appreciation to Mr. Tonkin for the preparation and distribution of the collaborative samples, to Mr. Steele for the preparation of the transmission curves, and to Mr. Earl K. Spotts of the Ward Baking Company for assistance in the preparation of this report.

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EFFECT OF ACID HYDROLYSIS ON THE RETROGRADATION OF AMYLOSE¹

ROY L. WHISTLER and CAROLYN JOHNSON²

ABSTRACT

Potato amylose retrogrades from aqueous solutions more slowly than amyloses from corn and wheat starches. On acid hydrolysis, the rate of retrogradation of potato amylose increases to a maximum and then continuously decreases. Corn amylose, though lying nearer the maximum rate of retrogradation, goes through a similar increase and decrease in rate of retrogradation when its molecular magnitude is decreased by hydrolysis. For both amyloses maximum retrogradation seems to occur at viscosities of η_{sp}/c of about 0.5.

Amylose from potato starch retrogrades in an aqueous solution at a slower rate than amylose from either corn or wheat starches. Retrogradation is the aggregation and partial crystallization of starch molecules. Solutions undergoing retrogradation may become opalescent, increase in cloudiness, increase in resistance to enzyme action, and decrease in viscosity. Amylose molecules, because of their linear nature, can coalesce and hence retrograde much more readily than the branched or bush-shaped amylopectin molecules. Even with amylose molecules, the crystallization is only partially complete, because of the steric difficulty and low probability in the attainment of perfect organization during the condensation of very long polymers. Entire molecules may sometimes crystallize in retrogradation, but it is more probable that for the most part only segments of molecules are oriented into a regular lattice to form regions of crystallinity which are capable of giving rise to a "B" x-ray pattern. Outside of these regions parts of molecules as well as entire molecules associate in more or less random fashion to produce amorphous regions. Several explanations may be given as to why potato amylose retrogrades more slowly than amylose of corn and wheat starches. One logical explanation is that the potato amylose molecules may be slightly branched, hence association is sterically hindered. Perhaps a better explanation is that the larger size of potato amylose molecules makes them so unwieldy that their

¹ Manuscript received August 16, 1948.

Paper presented before the Annual Meeting of the American Association of Cereal Chemists, Cincinnati, 1948. The subject matter of this paper has been undertaken in cooperation with the Committee of Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the War Department.

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rate of coalescence and aggregation is reduced. In either case slight hydrolytic degradation of the amylose should cause retrogradation to occur more rapidly.

The present work was undertaken to determine the effect of acid hydrolysis on the rate of retrogradation of amylose.

Materials and Methods

Amyloses. Amyloses were prepared by butanol fractionation (3, 4) of good quality commercial corn, wheat, and potato starches. All amyloses were recrystallized once by dissolution in hot butanol-saturated water to form a 1% solution of amylose and allowing the solution to cool to room temperature over a period of 48 hours. Usually precipitates were collected in a supercentrifuge (40,000 R.P.M.). A portion of the separated precipitate was dried and the remainder stored under butanol at 0°-5°. To convert the precipitate to a dry powder, it was vigorously stirred into ethanol (approximately one part by volume of precipitate to five parts by volume of ethanol); the mixture filtered; and the amylose thrice more treated with ethanol. The products on drying *in vacuo* over calcium chloride were colorless, fluffy powders.

The iodine-sorbing capacity of the amyloses was measured by the method of Bates, French, and Rundle (1) as modified by Wilson, Schoch, and Hudson (6) and by Whistler and Hilbert (5). Corn and wheat amyloses sorbed 200-203 mg. of iodine per g. of amylose. Potato amylose sorbed 208 mg. iodine per g.

Hydrolysis of Amylose. Solutions of amylose were prepared by adding amylose-butanol paste to boiling water and the mixture stirred at 95°-98° for about one hour to vaporize the butanol. Solutions were adjusted to contain 1% amylose. Each was poured into individual Pyrex bottles fitted with a reflux condenser and motor stirrer and was placed in a constant temperature bath at 96°. When the solution reached the temperature of the bath, 50% sulfuric acid was added to produce a normality of 0.005. After solutions had been hydrolyzed for definite periods, the acid was neutralized with 1 *N* sodium hydroxide. An excess of butanol was added and precipitation and isolation of the amylose performed in the usual way.

Determination of Retrogradation. Air-dried amylose was weighed into a 500 ml. Erlenmeyer flask in such amount as to produce a concentration of 0.85% in 450 ml. of solution. Forty milliliters of 1 *N* sodium hydroxide were added; the flask was flooded with nitrogen and the mixture stored overnight at 0°-2° to bring about complete dispersion of the amylose. The calculated amount of distilled water was then added and the alkali neutralized with 1 *N* sulfuric acid to a

pH of 6.0–6.5. Immediately after neutralization, aliquots of the dispersion were taken for determination of initial starch concentration. Starch content of the aqueous solutions was determined by a modification of the chromic acid oxidation method of Launer (2). The main portion of the amylose dispersion was placed in a 25° constant temperature bath. After 24 hours, 40–50 ml. were removed and centrifuged in covered cups for 10 minutes at 3,900 times gravity. The supernatant was carefully decanted through a coarse sintered glass funnel and aliquots taken for analysis of amylose content by the chromic acid oxidation method as indicated above. The per cent of amylose that retrograded was calculated as the difference from the initial amylose concentration.

Determination of Viscosity. Air-dry amylose was weighed into a 100 ml. volumetric flask in such amount as to produce a solution of 0.4% when the flask was filled to the mark. The amylose was dissolved by adding 50 ml. of 1 N sodium hydroxide, replacing the air in the flask by nitrogen, and allowing the mixture to stand at 0°. After 0–20 minutes the samples had dissolved.

The flask was warmed to 25° and filled to the mark with 1 N sodium hydroxide. A portion of the solution was filtered by gravity through a medium sintered glass funnel and 10 ml. of the filtrate pipetted into a Cannon-Ostwald-Fensky viscosity tube (No. 100) and the viscosity measured at 25°. At intervals of time, thereafter, the viscosity determination was repeated with fresh samples of solution from the volumetric flask. From a plot of reduced viscosity (η_{sp}/c) against time, a straight line was obtained which was extrapolated to zero time to give the initial viscosity of the amylose solution. To determine whether this method of extrapolation is justified, an investigation was made of the decrease in viscosity which alkaline-amylose solutions undergo with time. The results are shown in Fig. 1. A rapid decrease in reduced viscosity occurred when samples were stored in air or under nitrogen at 25°, but the decrease was slow when samples were stored at 0° under nitrogen. When the latter conditions are used, the data indicate that extrapolation to zero time is unnecessary if viscosity measurements are made soon after complete solution of the sample is attained. However, it was not always convenient to make measurements at this point, and the expedient of extrapolation to zero time was employed.

Results and Discussion

The rate of retrogradation of potato amylose is markedly affected by acid hydrolysis, as shown in Fig. 2. Unhydrolyzed amylose retrogrades from solutions of 0.85% concentration to the extent of only

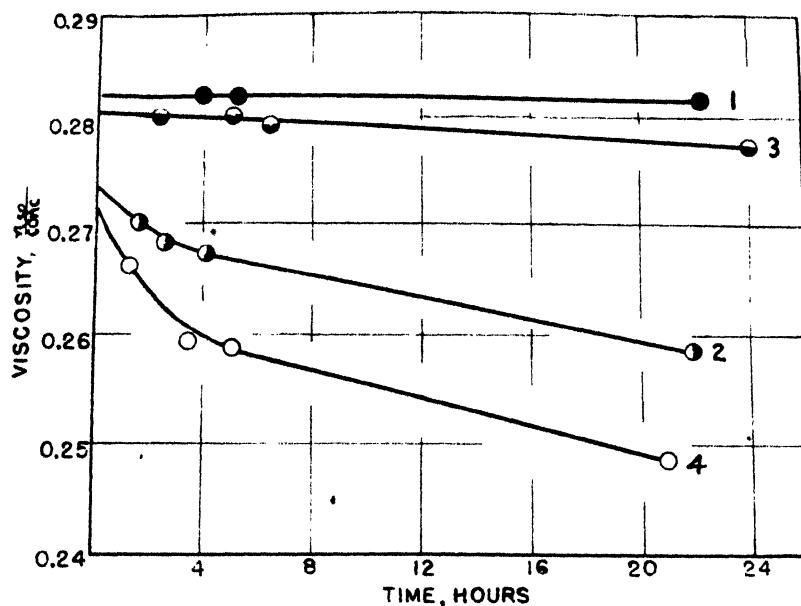


Fig. 1. Variation of viscosity with time for solutions of amylose (0.4%) in 1 N sodium hydroxide: (1) under nitrogen at 0°C., (2) under nitrogen at 25°C., (3) under air at 0°C., (4) under air at 25°C.

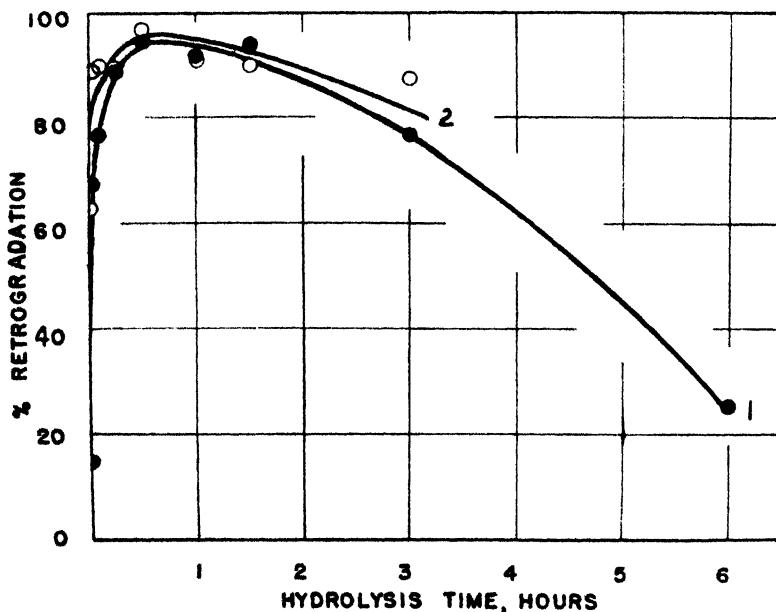


Fig. 2. Effect of acid hydrolysis on the extent of retrogradation at 25°C. (1) potato; (2) corn.

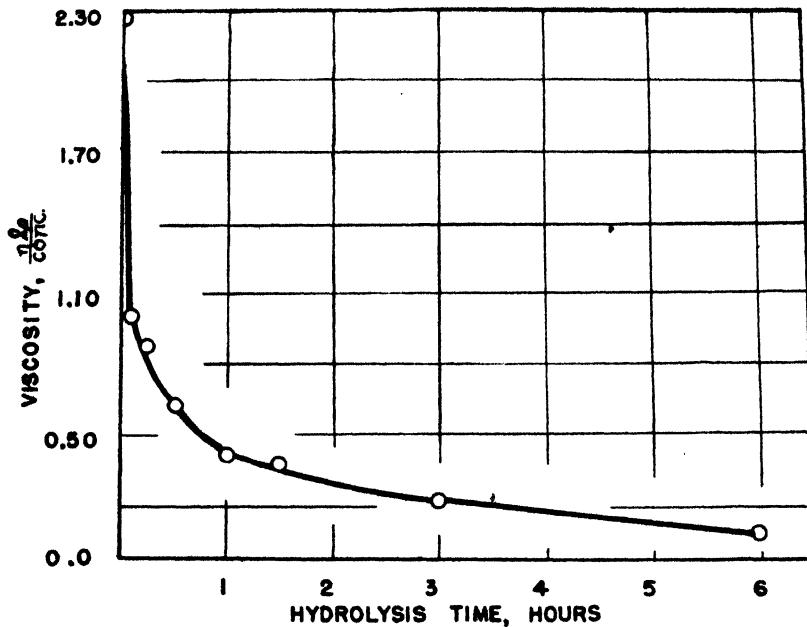


Fig. 3. Effect of time of acid hydrolysis on the viscosity of potato amylose at 25°C.

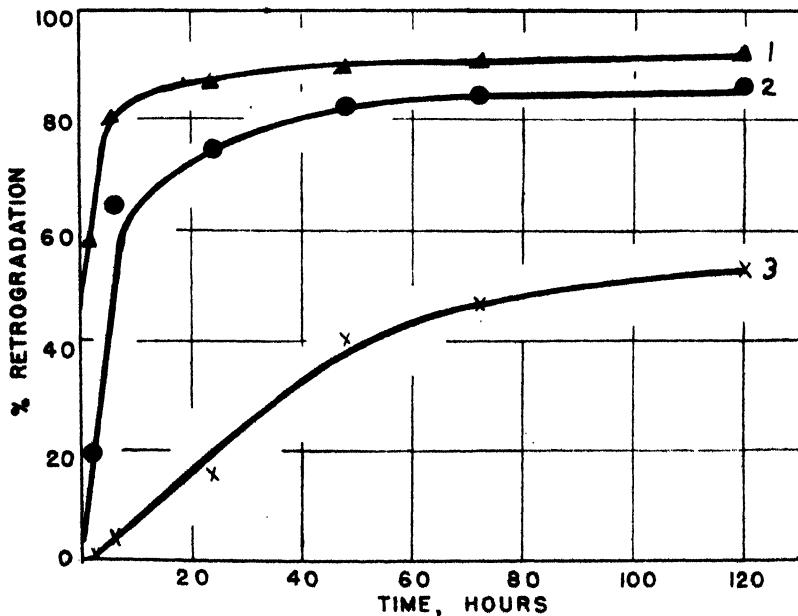


Fig. 4. Rate and extent of retrogradation of amyloses from 0.85% dispersions at 25°C. (1) wheat, (2) corn, (3) potato.

16% during the first 24 hours (see also Fig. 4). As acid hydrolysis proceeds, the rate of retrogradation increases until a maximum is reached and, thereafter, continuously decreases.

Maximum rates of retrogradation appear after 30 minutes to one hour of acid hydrolysis for the particular potato amylose used. After 6 hours of acid hydrolysis, the rate of retrogradation of the amylose again approaches the rate of retrogradation of the initial unhydrolyzed amylose.

During the hydrolysis period the molecular weight of the amylose decreases rapidly, as indicated by the viscosity data of Fig. 3. Samples which show maximum rates of retrogradation, that is, those isolated after 30 minutes to one hour of acid hydrolysis, have reduced viscosity values (η_{sp}/c) of 0.65 to 0.35. Amylose samples which have reduced viscosities greater than these values may be too large and clumsy to undergo rapid association. On the other hand, molecules with lower viscosity values have increased solubility due to their small size.

Amyloses from corn and wheat retrograde more rapidly than potato amylose, as indicated in Fig. 4. Under comparable conditions, corn and wheat amyloses retrograde to the extent of 75.4% and 86.7%, respectively, during the first 24 hours at 25°C. They do not retrograde as rapidly as potato amylose which has been hydrolyzed 30 minutes to one hour. Their reduced viscosities of 1.06 and 0.80 suggest that they may correspond to potato amylose samples on the ascending portion of the curve in Fig. 2. For example, a comparison of the reduced viscosity of corn amylose with the data in Fig. 3 indicates that the rate of retrogradation for this amylose could be increased to a maximum value by subjecting it to hydrolysis with 0.005 *N* sulfuric acid for a period of about 30 minutes. This would be sufficient time to lower the viscosity value to about 0.5 η_{sp}/c . When so hydrolyzed, the reduced viscosity of the isolated corn amylose is found to be 0.54. The extent of retrogradation in 24 hours is increased from 75.4% for the original unhydrolyzed amylose to 96.9% for the hydrolyzed sample. This latter value is comparable to the maximum rate of retrogradation for potato amylose (94%) exhibited in Fig. 2.

These experiments suggest that for amylose molecules there exists a critical size for which retrogradation rates are at a maximum. This maximum corresponds roughly to reduced viscosities of 0.45 to 0.56. Molecules of larger size retrograde more slowly, presumably because of steric effects resulting from their more coiled, kinked, or convoluted structure. Molecules of smaller size retrograde more slowly, presumably because of the increased solubility inherent in a lower molecular weight molecule.

This information may possibly prove of assistance to industrial workers who desire to maintain starch solutions with a minimum of retrogradation.

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COMMUNICATION TO THE EDITOR

Additional Data on Effect of Polyoxyethylene Stearate on the Crumb Softness of Bread

SIR:

In our paper, "Effect of polyoxyethylene stearate on the crumb softness of bread," *Cereal Chemistry* **24**: 346-355 (1947), the values in Table I and Fig. 1 show a substantial decrease in the rate of development of firmness of bread crumb containing this material. The data presented were obtained from tests of bread made on four different days.

Since this paper was submitted for publication, further tests have been conducted over the period February, 1947, to June, 1948, inclusive, in which bread made by the same formula and procedure as that used in the above paper, with and without the addition of 0.5% polyoxyethylene stearate, was made and tested for crumb softness at one and three days of age, as described in the paper. Comparative data for 53 pairs of observations were available and have been submitted to statistical analysis by Dr. James C. Munch, Upper Darby, Pennsylvania. Excerpts from Dr. Munch's unpublished report are submitted herewith as substantiation of the data originally presented.

"To obtain the values for the mean (\bar{X}) we have added together the 53 individual values and divided by 53, giving the four results shown. . . . The standard deviation, sigma \bar{X} , follows: then CV which represents sigma as percent of mean, for the N or 53 measurements.

"Application of the 't' test to these data indicated that there was no significant difference between the mean hardness of bread with and without 0.5% polyoxyethylene stearate after one day. On the other hand, bread made with the P.O.E.S. was significantly softer than the

TABLE I

STATISTICAL SUMMARY OF FIRMNESS OF BREAD MADE WITH AND WITHOUT
0.5% (FLOUR BASIS) POLYOXYETHYLENE STEARATE AS
MEASURED BY THE BAKER COMPRESSIMETER

	Firmness as stress per 2.5 mm. strain			
	Control		0.5% polyoxyethylene stearate	
	1 day old	3 days old	1 day old	3 days old
Mean (\bar{X})	96.17	185.30	83.13	143.42
Sigma \bar{X}	3.38	5.70	2.46	4.68
CV	3.5	3.1	3.0	3.3

control bread after three days. In the case of both breads there was a significant increase in firmness of crumb as the bread aged from one to three days and the rate of increase of the bread containing P.O.E.S. was significantly less than that of the control bread."

The average values obtained in this study compare closely with those previously reported.

Further work on the baking characteristics of this material indicates that it does have considerable dough conditioning and improving effect when used with certain types of flour not covered in the original paper. This is being investigated more thoroughly.

H. H. FAVOR and N. F. JOHNSTON

R. T. Vanderbilt Co., Inc.

East Norwalk, Connecticut

BOOK REVIEW

Cottonseed and Cottonseed Products. Their chemistry and chemical technology.
Edited by Alton E. Bailey. 936 pp. Interscience Publishers, Inc., New York,
N. Y. 1948. Price \$17.50.

This book is a comprehensive treatise on the chemistry and technology of the cottonseed industry and will be the most important single source of reference for research and development workers in this field. Every important development in the cottonseed industry is discussed or at least referred to.

The book represents a tremendous accomplishment by the editor and 24 other eminent authorities who have contributed to the 5 sections and the 24 chapters. The complete coverage of the subject makes this a book to be referred to and studied by chapter rather than to be read completely through. A number of the chapters and substantial portions of others are of general interest and can be read easily and profitably by anyone desiring a general background of the cottonseed industry. All of the contributing authors are qualified, if not outstanding, authorities in the fields assigned to them. The editor has done an outstanding job in exercising close editorial supervision to maintain a uniformity of treatment with a minimum of duplication. The text is liberally illustrated and supplemented by charts, tables, flow sheets, photographs, selected group bibliographies, and references which exhaustively cover the subject of cottonseed and its products.

In summarizing and correlating the past and current technical achievements in the industry, several authors have also given excellent focus and direction to future needed development. Any organization or individual undertaking a research program on cottonseed or its products will do well to study the sections of this book that point out the most profitable field of additional study.

The history of the cottonseed industry makes fascinating reading. It is an industry with many unique characteristics. Although the technical advancements have been tremendous, the production of crude oil is still more an art than a science. As everyone who is engaged in it knows, he and his fellow workers comprise the finest group of people in the world. Few industries retain the rough and tough competition that still exists between operators, but few contain the fellowship and personal warm friendships that are the rule in this industry. For this reason, the development of the various trade associations amply covered in the book has an unusually important bearing on the development of the industry itself.

A listing of the sections and chapters together with the authors seems to be the only way to conclude a review of this book within a reasonable space.

A. History and Present Status of the Cottonseed Industry

- I. History of Cottonseed and the United States Cottonseed Industry. By Maurice R. Cooper.
- II. Production and Consumption of Cottonseed and Cottonseed Products. By Charles E. Lund.

B. Composition and Characteristics

- III. Structure of the Cottonseed. By John Leahy.
- IV. Cottonseed Composition—Relation to Variety, Maturity and Environment of the Plant. By W. H. Tharp.
- V. Biological Processes of the Cottonseed. By Aaron M. Altschul.
- VI. Pigments of Cottonseed. By Charlotte H. Boatner.
- VII. Cottonseed Oil. By A. E. Bailey.
- VIII. Cottonseed Protein. By Thomas D. Fontaine.
- IX. Miscellaneous Constituents. By F. G. Dollear and K. S. Markley.

C. Grading and Evaluation of Cottonseed and Its Primary Products

- X. Grading and Evaluation of Cottonseed. By Guy S. Meloy.
- XI. Grading and Evaluation of Cottonseed Oil, Cake and Meal. By E. R. Barrow.
- XII. Grading of Cotton Linters. By Guy S. Meloy.

D. Cottonseed Processing

- XIII. Handling and Storing of Cottonseed. By O. H. Alderks.
- XIV. Mechanical Pretreatment of the Seed. By A. C. Wamble.
- XV. Cooking of Meats and Recovery of the Oil. By O. H. Alderks.
- XVI. Economics of Cottonseed Crushing. By J. F. Moloney.

E. Utilization of Cottonseed Products

- XVII. Processing of Cottonseed Oil. By Edward M. James.
- XVIII. Edible Cottonseed Oil Products. By Howard C. Black.
- XIX. Nutrition Aspects of Cottonseed Oil Utilization—The Role of Fat in Human Nutrition. By Harry J. Deuel, Jr.
- XX. Nonedible Cottonseed Oil Products. By O. H. Wurster, W. J. Govan, G. J. Stockmann.
- XXI. Cottonseed as a Source of Animal Feedstuffs. By Fred Hale and Carl M. Lyman.
- XXII. Miscellaneous Products from Seed and Meal. By A. E. Bailey.
- XXIII. Cottonseed Hulls. By John W. Dunning.
- XXIV. Cottonseed Linters. By Peter Van Wyck.

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SUGGESTIONS TO AUTHORS

General. From January 1, 1948, an abstract will be printed at the beginning of each paper instead of a summary at the end, references will be numbered to provide the option of citing by number only, and date of receipt, author's connections, etc., will be shown in footnotes. Except on these points, authors will find the last volume of *Cereal Chemistry* a useful guide to acceptable arrangements and styling of papers. "On Writing Scientific Papers for Cereal Chemistry" (*Trans. Am. Assoc. Cereal Chem.* 6: 1-22, 1948) amplifies the following notes.

Authors should submit two copies of the manuscript, typed double spaced with wide margins on 8½ by 11 inch white paper, and all original drawings or photographs for figures. If possible, one set of photographs of figures should also be submitted. Originals can then be held to prevent damage, and the photographs can be sent to reviewers.

Titles and Footnotes. Titles should be specific, but should be kept short by deleting unnecessary words. The title footnote shows "Manuscript received . . ." and the name and address of the author's institution. Author footnotes, showing position and connections, are desirable although not obligatory.

Abstract. A concise abstract of about 200 words follows title and authors. It should state the principal results and conclusions, and should contain, largely by inference, adequate information on the scope and design of the investigation.

Literature. In general, only recent papers need be listed, and these can often be cited more advantageously throughout the text than in the introduction. Long introductory reviews should be avoided, especially when a recent review in another paper or in a monograph can be cited instead.

References are arranged and numbered in alphabetical order of authors' names and show author, title, journal, volume, first and last pages, and year. The list is given at the end of the paper. Reference numbers must invariably be cited in the text, but authors' names and year may be cited also. Abbreviations for the names of journals follow the list given in *Chemical Abstracts* 40: I-CCIX. 1946.

Organization. The standard organization involves main sections for abstract, introduction, materials, methods, results, discussion, acknowledgments, and literature cited. Alternately, a group of related studies, each made with different materials or methods, may require a separate section for each study, with subsections for materials and methods, and for results, under each section. Center headings are used for main sections and italicized run-in headings for subsections, and headings should be restricted to these two types only.

Tables. Data should be arranged to facilitate the comparisons readers must make. Tables should be kept small by breaking up large ones if this is feasible. Only about eight columns of tabular matter can be printed across the page. Authors should omit all unessential data such as laboratory numbers, columns of data that show no significant variation, and any data not discussed in the text. A text reference can frequently be substituted for columns containing only a few data. The number of significant figures should be minimized. Box and side headings should be kept short by abbreviating freely; unorthodox abbreviations may be explained in footnotes, but unnecessary footnotes should be avoided. Leader tables without a number, main heading, or ruled lines are often useful for small groups of data.

Tables should be typed on separate pages at the end of the manuscript, and their position should be indicated to the printer by typing "(TABLE I)" in the appropriate place between lines of the text. (Figures are treated in the same way.)

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Text. Clarity and conciseness are the prime essentials of a good scientific style. Proper grouping of related information and thoughts within paragraphs, selection of logical sequences for paragraphs and for sentences within paragraphs, and a skillful use of headings and topic sentences are the greatest aids to clarity. Clear phrasing is simplified by writing short sentences, using direct statements and active verbs, and preferring the concrete to the abstract, the specific to the general, and the definite to the vague. Trite circumlocutions and useless modifiers are the main causes of verbosity; they should be removed by repeated editing of drafts.

Editorial Style. A.A.C.C. publications are edited in accordance with *A Manual of Style*, University of Chicago Press, and *Webster's Dictionary*. A few points which authors often treat wrongly are listed below:

Use names, not formulas, for text references to chemical compounds. Use plural verbs with quantities (6.9 g. were). Figures are used before unit abbreviations (3 ml.), and % rather than "per cent" is used following figures. All units are abbreviated and followed by periods, except units of time, which are spelled out. Repeat the degree sign (5°-10° C.). Place 0 before the decimal point for correlation coefficients ($r = 0.95$). Use * to mark statistics that exceed the 5% level and ** for those that exceed the 1% level; footnotes explaining this convention are no longer required. Type fractions on one line if possible, e.g., $A/(B + C)$. Use lower case for farinograph, mixogram, etc., unless used with a proper name, i.e., Brabender Farinograph. When in doubt about a point that occurs frequently, consult the Style Manual or the Dictionary.

CORRECTION

Magnification for Fig. 1, page 315 (September 1948)
should read $\times 540$.

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